FUNCTIONAL SIGNIFICANCE OF SEQUENCE VARIATION AMONG MIR-200/205 FAMILIES OF MIRNAS IN OVARIAN CANCER

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FUNCTIONAL SIGNIFICANCE OF SEQUENCE VARIATION AMONG MIR-200/205 FAMILIES OF MIRNAS IN OVARIAN CANCER

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To my family

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LIST OF ABBREVIATIONS

EMT	Epithelial-Mesenchymal Transition
MET	Mesenchymal-Epithelial Transition
miRNA	MicroRNA
OC	Ovarian Cancer
TYMS	Thymidylate Synthetase
ANXA4	Annexin A4
Cul4A	Cullin4A
FOXO1	Forkhead Box O1
Rac1	Ras-Related C3 Botulinum Toxin Substrate 1
GSN	Gelsolin

SUMMARY

MicroRNAs are small RNAs that regulate large suites of target genes. Recent findings have implicated members of the miR-200 family of miRNAs in the epithelial-mesenchymal/mesenchymal-epithelial (EMT/MET) process that is associated with cancer metastasis. In chapter 2, our results indicate that significant variation exists among miR-200 miRNAs in the regulation of molecular processes underlying specific features of the EMT/MET process and that this variation is associated with sequence variation mapping to both the seed and non-seed region of individual family members. Our results generally support the notion that modulations in the expression of miRNAs involved in EMT may serve as the basis of new strategies in the treatment of cancer. In chapter 3, we present a set of analyses on the functional and evolutionary significance of human miRNA seed region variations. We provide computational and experimental evidence that as little as a single nucleotide change within seed regions dramatically alters the spectrum of mRNAs directly or indirectly regulated by the over expressed miRNA and that additional seed nucleotide substitutions have no significant added effect. Our results help explain the highly conserved nature of seed regions among many miRNA families within and across species and develop a better understanding of the evolution of miRNA mediated regulation. In chapter 4, we provide evidence for seed/non-seed region interactions in the regulation of miRNA-induced cisplatin resistance in ovarian cancer cells.

CHAPTER 1

INTRODUCTION

Background

Cancer is a major burden of disease worldwide. Chances of survival are greater if cancer is diagnosed when still confined to the organ of origin, however, survival rates decline as tumors spread and metastasize to distant organs. During metastasis, cells initially acquire properties that allow at least partial separation from the original mass to escape through boundary matrices and intravasate into a conduit vessles (Wells, Chao et al. 2011) . At the metastatic site, the cells exit the blood or lymphatic vessels, form micrometastatic nodules, adapt and reprogram the surrounding stroma, and eventually form macrometastases (Yao, Dai et al. 2011). Both differentiated primary tumors and metastases often have a similar heterogeneous organization that is characterized by regions of dedifferentiation, particularly at the invasive front. This de-differentiation is a hallmark of an Epithelial–Mesenchymal Transition (EMT)(Brabletz 2012). Once epithelial cells that display a highly baso-apical polarization (Voulgari and Pintzas 2009) become competent to respond to EMT-inducing signals, the signals can promote loss of this polarity, disruption of the intercellular adhesion complexes (Moreno-Bueno, Portillo et al. 2008) and a complete reorganization of the actin cytoskeleton (Yilmaz and Christofori 2009). Simultaneously, the cells acquire migratory and invasive properties that allow them to migrate through the extracellular matrix and disseminate from the primary tumor (Acloque, Adams et al. 2009, Brabletz 2012). The disseminated cells recruited to the target organs may re-acquire epithelial characteristics similar to the

primary tumor and undergo a reverse phenotypic transition from mesenchymal to epithelial by a process called Mesenchymal- Epithelial Transition (MET) (Thiery and Sleeman 2006). This latter change may be triggered by the local environment after extravasation towards the parenchyma of the invaded organs (Foroni, Broggini et al. 2012).

The EMT regulatory networks are closely connected, and modulation of any of these networks has a profound effect on every other network (Craene and Berx 2013). The most extensively studied network is built around EMT inducing transcription factors that repress E-cadherin, a major component of adherens junctions and hallmark of the epithelial integrity (Peinado, Olmeda et al. 2007), and also simultaneously repress several other junctional proteins, which facilitate EMT (Ikenouchi, Matsuda et al. 2003, Vandewalle, Comijn et al. 2005). Besides the well-established transcriptional reprogramming during EMT, post-transcriptional mechanisms such as small non-coding RNAs known as miRNAs provide an additional layer of gene regulation (Craene and Berx 2013, Lamouille, Subramanyam et al. 2013). In the next sections, we summarize our findings on the effectiveness of a panel of miRNAs on the induction of hallmarks of MET in ovarian cancer cells, functional and evolutionary significance of sequence variation among miRNAs and provide evidence for the functionally significant interactions between seed and non-seed regions among members of the miR-200 family.

Regulation of EMT by miRNAs

MiRNAs are endogenous non-coding RNAs (~22 nt) that regulate gene expression at the post-transcriptional level (Bartel 2004). MiRNA target recognition is primarily determined by pairing of the highly conserved miRNA seed sequence

(nucleotides2–8) to complementary sites in target mRNAs (Lewis, Shih et al. 2003, Friedman, Farh et al. 2009). Many miRNAs have been directly or indirectly associated with EMT (Zhang and Ma 2012). The miR-200 family (miR-200a, miR-200b, miR-429, miR-200c and miR-141) is a widely known miRNA family that is strongly associated with epithelial differentiation regulating both EMT and MET (Gregory, Bert et al. 2008, Park, Gaur et al. 2008).

In chapter 2, we demonstrate the effectiveness of the members of the miR-200 family to induce MET in ovarian cancer cells. It has been previously shown that a well characterized mesenchymal-like OC cell line (HEY) undergoes MET in response to ectopic over-expression of miR-429 (Chen, Wang et al. 2011). In the study presented in chapter 2, we first determined that the ability of miR-429 to induce MET extends to other mesenchymal-like OC cell lines, HEY A8 (Mills, May et al. 1990) and SKOV-3 (Fogh, Fogh et al. 1977, Lis, Touboul et al. 2014). We next verified that ectopic over expression of each member of the miR-200 family induces significant changes in many of the morphological and molecular hallmarks that are associated with MET in HEY OC cell lines. More importantly, we found that significant differences exist among these members in the expression of a panel of epithelial and mesenchymal biomarkers and in induced cisplatin sensitivity. Interestingly, this functional variability was associated with variation mapping to the seed and non-seed regions of individual miRNAs.

In chapter 3, we present a set of computational and experimental analyses on the functional and evolutionary significance of human miRNA seed region mutations. Initially, the relationship between seed sequence variation and miRNA target overlap across all conserved miRNAs was assessed using miRNA target prediction tools. The

analyses indicated that as few as one nucleotide change within the 7-mer miRNA seed region (nucleotides 2–8 at the 5′-end of the miRNA) dramatically alters the spectrum of targeted mRNAs. Additionally, computational analyses predicted that additional seed nucleotide substitutions have little to no additional effect on miRNA targeting. We next present experiments that evaluate the computationally driven results and test the consequence of seed region variations on patterns of gene expression. In order to do so, we conducted ectopic over expression of synthetic miRNAs with variable seed regions but with identical non-seed regions. Consistent with our computationally assessed results, the experimental data again demonstrate that as few as one nucleotide change within seed regions dramatically alters the spectrum of mRNAs directly or indirectly regulated by the over expressed miRNA and additional seed nucleotide substitutions (5 nt) have no significant additional effect. Our results help explain the highly conserved nature of seed regions among many miRNA families within and across species and develop a better understanding of the evolution of miRNA mediated regulation.

In chapter 2, we have demonstrated that over-expression of miR-200b in mesenchymal-like ovarian cancer cells significantly increases sensitivity to the chemotherapeutic drug cisplatin and that this functionally significant change is associated with sequence variation in the non-seed region on miR-200b. In chapter 4, we report that the miR-200b induced increase in drug sensitivity is correlated with significant gene expression changes including the down-regulation of genes known to be associated with cisplatin resistance in cancer cells. We further demonstrate evidence that upon change of a single nucleotide within the miR-200b seed region, its ability to impart cisplatin sensitivity is lost. Our results provide an example on the significance of seed /non-seed

interactions in the regulation of miRNA-induced cisplatin resistance in ovarian cancer cells.

CHAPTER 2

SEQUENCE VARIATION AMONG MEMBERS OF THE MIR-200 MICRORNA FAMILY IS CORRELATED WITH VARIATION IN THE ABILITY TO INDUCE HALLMARKS OF MESENCHYMAL-EPITHELIAL TRANSITION IN OVARIAN CANCER CELLS

Preliminary comments

The contents in this chapter are published in the Journal of Ovarian Research under the title "Sequence variation among members of the miR-200 microRNA family is correlated with variation in the ability to induce hallmarks of mesenchymal-epithelial transition in ovarian cancer cells". I am first author on this paper and I have performed all the experiments and data analyses in the study. Ashley Reavis has assisted with the experiments on HEY A8 and SKOV-3 cells.

Abstract

Background

Epithelial-Mesenchymal Transition (EMT) is a transient and reversible (MET) process by which epithelial cells acquire mesenchymal, fibroblast-like cell characteristics including reduced intercellular adhesion and increased cell motility. While EMT/MET has long been recognized as an essential component of early embryonic development, there is a growing body of evidence indicating that EMT/MET also plays a key role in ovarian cancer metastasis. Recent findings have implicated members of the miR-200 family of microRNAs (miRNAs) in this process.

Methods

Individual members of the miR-200 family of miRNAs were transiently overexpressed in metastatic (mesenchymal-like) OC cell lines. Changes in morphology, molecular profiles and drug sensitivity were monitored relative to cells transfected with a negative control miRNA.

Results

Morphological hallmarks of MET were detected in cells transfected with all members of the miR-200 family. Gene expression profiling demonstrated up regulation of epithelial biomarkers and down regulation of mesenchymal biomarkers in transfected cells although significant variation in molecular response and drug sensitivity was associated with different miR-200 family members.

Conclusions

Our results indicate that although ectopic overexpression of all members of the miR-200 family in mesenchymal-like OC cells results in morphological changes characteristic of MET, the underlying molecular changes and induced drug sensitivities are highly variable and correlated with sequence variation within the seed and non-seed regions of individual miR-200 family members.

Background

Ovarian cancer is the most lethal of all gynecologic malignancies (Siegel, Ward et al. 2011). The majority of ovarian cancer related deaths is attributable to the spread of cancer cells from the primary tumor to metastatic sites throughout the abdominal cavity (Halkia, Spiliotis et al. 2012). During early stages of metastasis, a subset of primary

epithelial tumor cells undergo Epithelial-Mesenchymal Transition, whereby cell-cell adhesion complexes are disrupted, the characteristic apico-basal polarity of the cells is lost and cells acquire elevated levels of motility, invasiveness and resistance to standard chemotherapeutic agents (Huber, Kraut et al. 2005, Acloque, Adams et al. 2009, Brabletz 2012). Following attachment to secondary sites, metastasizing cells undergo a complementary Mesenchymal-Epithelial Transition whereby the metastatic cells reacquire epithelial morphology and other features characteristic of the primary tumor cells (Chaffer, Thompson et al. 2007). Because of the high clinical significance of metastasis, considerable effort is currently being directed towards the development of new classes of agents that may reduce the spread of cancer cells by inducing MET (Monteiro and Fodde 2010).

We have previously shown that mesenchymal-like ovarian cancer cells undergo MET in response to ectopic overexpression of miR-429, a member of the miR-200 family of miRNAs (Chen, Wang et al. 2011). This finding is consistent with earlier observations implicating members of the miR-200 miRNA family in MET (Gregory, Bert et al. 2008, Park, Gaur et al. 2008). Here, we report the results of a systematic examination of the effects of ectopic overexpression of members of the miR-200 family in ovarian cancer mesenchymal-like cell lines. The results indicate that although overexpression of each member of the miR-200 family induces significant changes in many of the morphological and molecular hallmarks of MET, significant differences exist among family members in the expression of EMT/MET biomarkers and in induced drug sensitivity. This functional variability is associated with sequence variation mapping to both the seed and non-seed regions of individual miRNAs.

Results

MiR-429 is capable of inducing Mesenchymal-Epithelial Transition in multiple mesenchymal-like ovarian cancer cell lines

We have previously shown that ectopically overexpressed miR-429 induces MET in a well-characterized mesenchymal-like OC cell line (HEY) (Chen, Wang et al. 2011). We were interested in determining if this ability of miR-429 to induce MET extends to other mesenchymal-like ovarian cancer cell lines as well. Consistent with our previous results, ectopically overexpressed miR-429 in two additional mesenchymal-like OC cell lines (HEY A8 (Mills, May et al. 1990) and SKOV-3 (Fogh, Fogh et al. 1977, Lis, Touboul et al. 2014)) induced morphological and molecular changes consistent with MET (Figure 2.1). MiR-429 transfected SKOV-3 and HEY A8 cells displayed a change from the elongated and fibroblast like shape characteristic of mesenchymal cells to the more rounded shape characteristic of epithelial cells. This morphological change was objectively validated using the CellProfiler cell image analysis software (Carpenter, Jones et al. 2006). Changes in expression of two epithelial KRT8 and KRT18 and two mesenchymal [ZEB1 and ZEB2 (zinc finger E-box binding homeobox 1 and 2)] molecular biomarkers were consistent with MET.

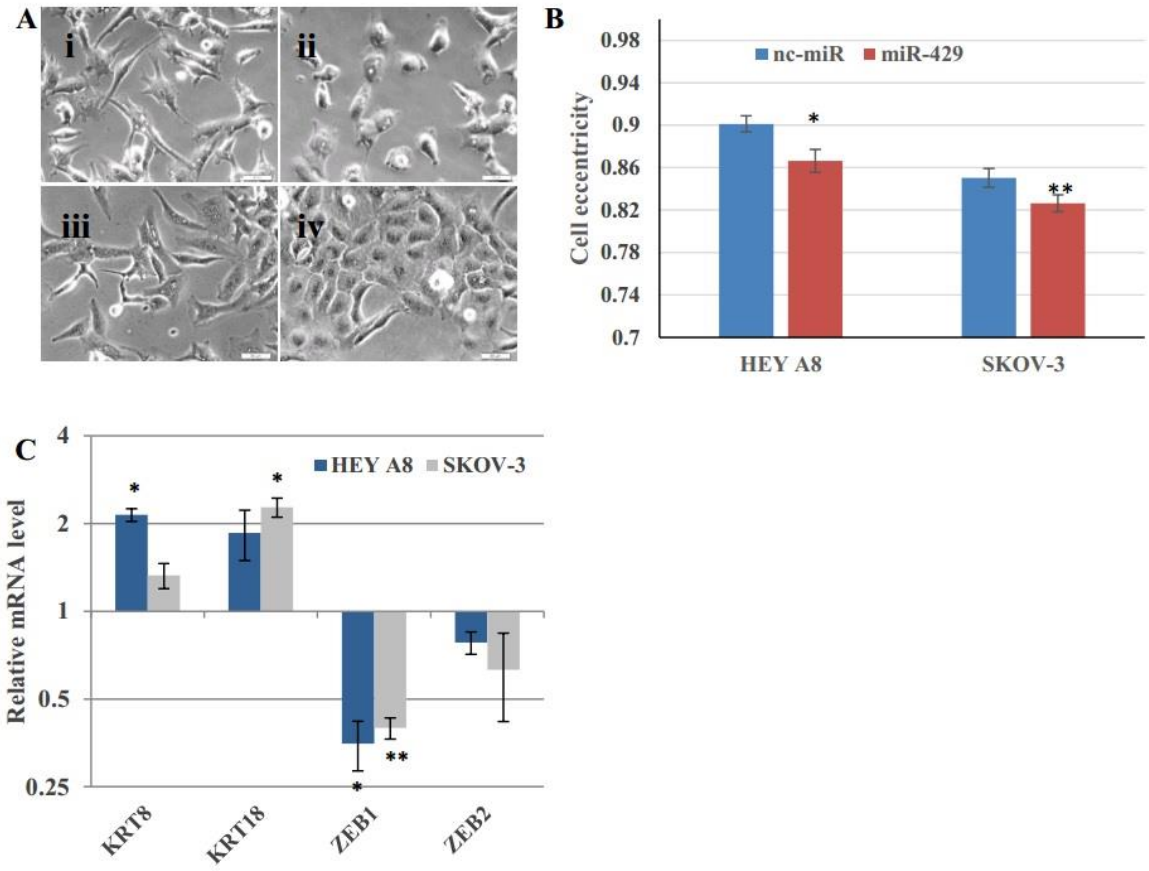


Figure 2.1. Overexpression of miR-429 in SKOV-3 and HEY A8 cells induces morphological and molecular changes consistent with the induction of Mesenchymal Epithelial Transition. (A) Representative phase contrast microscopy images of HEY A8 cells transfected with (i) nc-miR and (ii) miR-429, and SKOV-3 cells transfected with (iii) nc-miR and (iv) miR-429, 48 hours post transfection. Scale bars indicate 50 μm. (B) The accumulation of rounded epithelial-like cells occurred in miR-429 transfected groups (eccentricity: a score of 0 = circular shape, a score of 1 = linear shape). Values represent mean ± SEM. Mann-Whitney U test was used to detect significance of differences between miR-429 and negative control groups (*P <0.05, **P <0.005, n = 127-258 cells analyzed per group). (C) Relative mRNA expression of representative epithelial and mesenchymal biomarkers. Expression values in miR-429 transfection are normalized to negative control in each cell line and represent mean ± SEM of three biological replicates each performed in three technical replicates. Student's t-test was used to detect significance of differences between miR-429 and negative control group in each cell line (*P <0.005, **P < 0.0005,).

All members of the miR-200 miRNA family are capable of inducing Mesenchymal-Epithelial Transition in ovarian cancer mesenchymal-like cells

All members of the miR-200 family have previously been either directly or indirectly implicated in EMT (Gregory, Bert et al. 2008, Park, Gaur et al. 2008). Having established that ectopic over expression of miR-429, a member of the miR-200 miRNA family (Figure 2.2A), induces MET in a variety of mesenchymal-like OC cell lines, we were interested in determining if this MET-inducing potential extends to other members of the miR-200 family as well.

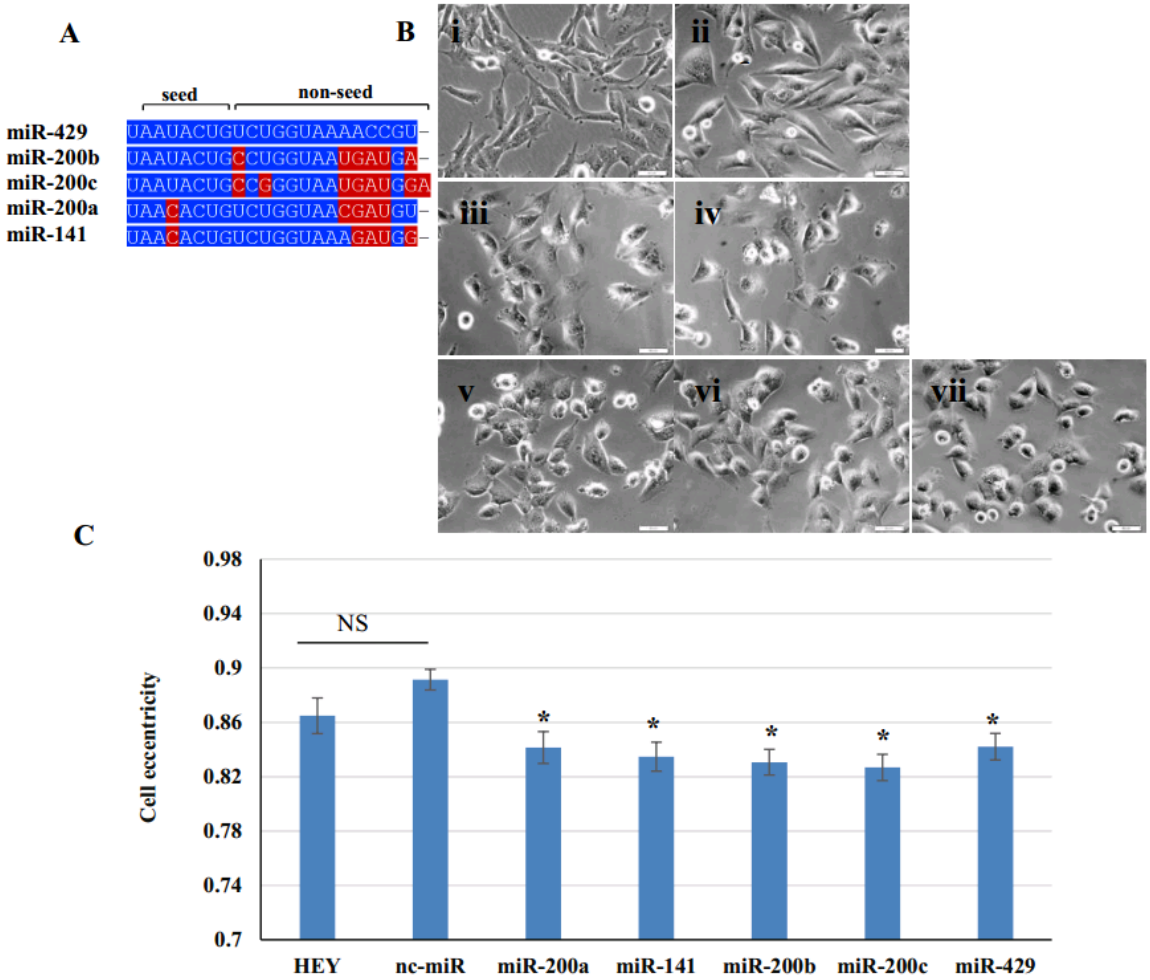


Figure 2.2. Overexpression of the miR-200 family induces morphological changes consistent with the induction of MET. (A) Multiple sequence alignment of the miR-200 family. Alignment is performed using the ClustalW2 program. Sequence differences relative to miR-429 are highlighted. (B) Representative phase contrast microscopy images of (i) HEY cells (not transfected), HEY cells transfected with (ii) nc-miR, (iii)

miR-200a, (iv)miR-141, (v) miR-200b, (vi) miR-200c, and (vii) miR-429, 48 hours post transfection. Scale bars represent 50 μm. (C) The accumulation of rounded epithelial-like cells occurred in miR-200 family transfected cells. Values represent mean ± SEM (n = 124-211 cells analyzed per group). Mann-Whitney U test is used to detect significant differences from the negative control (*P <0.0001, NS = not significant).

The results in Figure 2.2 demonstrate that overexpression of all miR-200 family members results in a significant change from the elongated, spindle-shaped morphology of the mesenchymal-like cells to the more rounded, cuboidal morphology characteristic of epithelial cells. No detectable change in morphology was observed in cells treated with the negative control (nc-miR).

The expression levels of a series of previously established epithelial and mesenchymal biomarkers (Zeisberg and Neilson 2009) were monitored in HEY cells transfected with members of the miR-200 family. Consistent with acquisition of a more epithelial phenotype, expression levels of all of the epithelial biomarkers (KRT8, KRT18, KRT7) were increased after overexpression of each member of the miR-200 family (Figure 2.3). As expected, the mesenchymal biomarkers ZEB1/ZEB2 displayed a general reduction in expression levels after overexpression of each member of the miR-200 family. The mesenchymal biomarker FN1 was also significantly down regulated in cells transfected with miR-200b, miR-200c and miR-429.

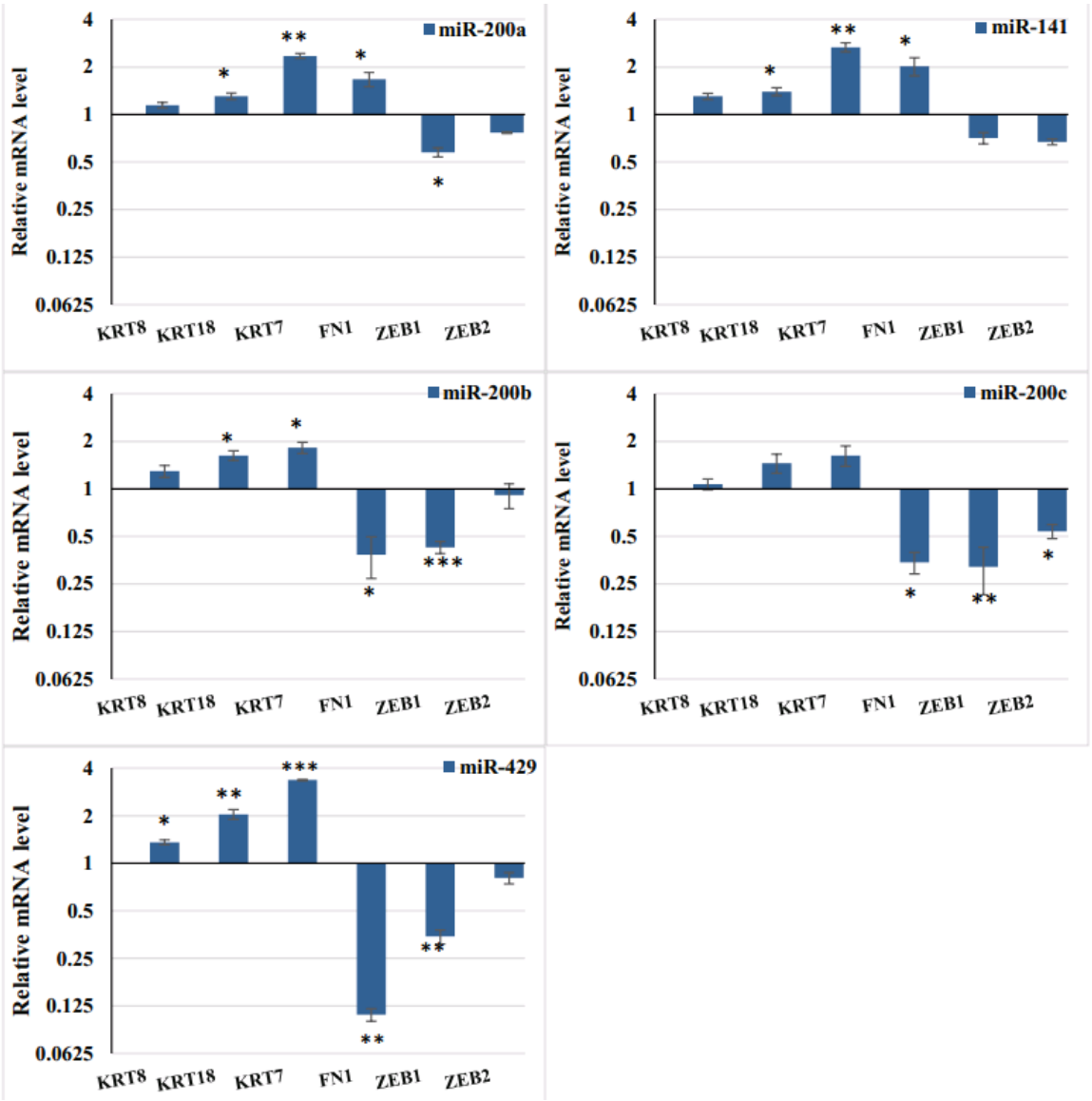


Figure 2.3. Overexpression of the miR-200 family induces changes in biomarkers consistent with the induction of MET. Expression values of representative epithelial and mesenchymal biomarkers after overexpression of miR-200 family members in HEY cells are normalized to negative control (nc-miR) and represent mean ± SEM of at least three biological replicates each performed in three technical replicates. Student's t-test is used to detect significant differences from the nc-miR transfection (*P <0.05, **P <0.005, ***P <0.0005).

Human miRNAs are known to be capable of regulating expression of their target genes by modulating mRNA levels and/or blocking translation (Bartel 2004). Thus, to further explore the inconsistent response of changes in FN1 RNA levels after transfection

by individual members of miR200 family, we additionally monitored expression changes on the protein level by immunofluorescence staining. The epithelial biomarker, CDH1 (Takeichi 1990, Zeisberg and Neilson 2009), was included in these assays as an additional control (Figure 2.4).

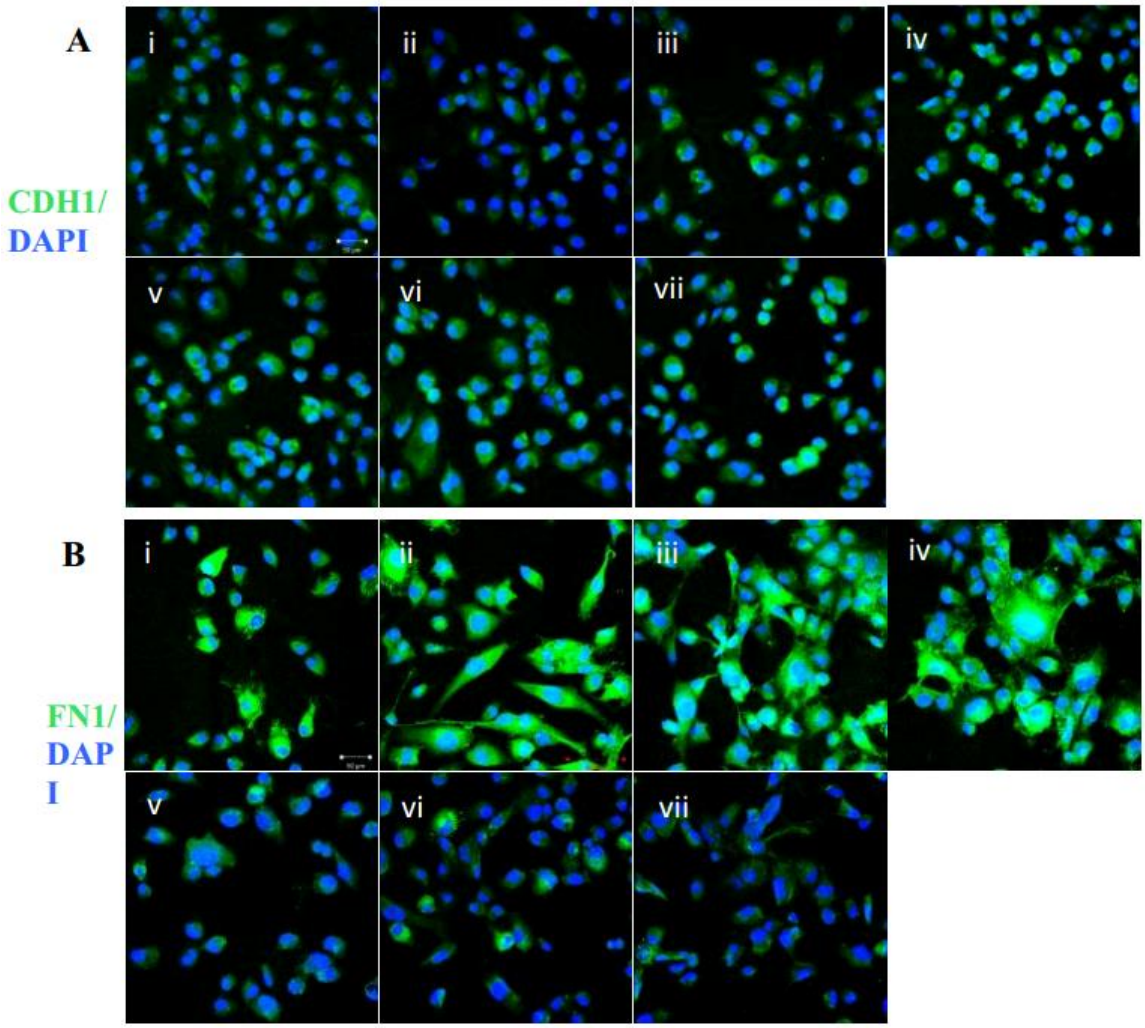


Figure 2.4. Immunostaining of cells for CDH1 (A) and FN1 (B) proteins. (i) HEY cells (not transfected), HEY cells transfected with (ii) nc-miR, (iii) miR-200a, (iv)miR-141, (v) miR-200b, (vi) miR-200c, and (vii) miR-429. DAPI was used to stain the cell nuclei. Scale bars represent 50 μm.

Consistent with the observed increase in expression levels of each of the epithelial biomarkers monitored on the RNA level, CDH1 protein was detected in all transfected cells but was absent in un-transfected cells and in cells transfected with the negative control. Interestingly, levels of FN1 protein were generally consistent with what was observed on the RNA level, i.e., protein levels were significantly down regulated in cells transfected by miR-200b, miR-200c or miR-429 but unchanged or slightly up-regulated in cells transfected by miR-200a, miR-141 (Figure 2.4). Collectively, these findings suggest that FN1 is a regulatory target of miR-200b, miR-200c and miR-429 but is not subject to regulation by miR-200a and miR-141. In addition, since a change from a mesenchymal to an epithelial phenotype was observed in cells transfected by all members of the miR-200 family, we conclude that FN1 is non-essential for MET in OC.

Previous studies indicate that the most functionally significant component of miRNA/mRNA pairing involves the miRNA "seed region", i.e., nucleotides 2-8 on the 5′ end of the miRNA (Lewis, Shih et al. 2003). Although all members of the miR-200 family are sequentially distinct, miR-200b, miR-200c and miR-429 are sequentially identical within their respective seed regions (Figure 2.2A). This identity may explain their shared ability to down regulate FN1 when ectopically over expressed in HEY cells. To further explore this possibility, we utilized three miRNA target prediction algorithms (miRanda (Betel, Wilson et al. 2008), miRDB (Wang 2008), and TargetScan (Lewis, Burge et al. 2005)) to identify members of the miR-200 family that are predicted to directly target FN1 mRNA and the other biomarkers monitored in our PCR experiments. The results, presented in Table 2.1, indicate that all three algorithms predict the presence of binding sites in FN1 mRNA for miR-200b, miR-200c and miR-429, but not for miR-

200a, miR-141. These computational predictions are consistent with our experimental results and indicate that differences in target specificity for FN1 mRNA between the miR-200 family members accounts for the observed variation in regulation of FN1 expression.

Table 2.1. Prediction of miR-200 family binding sites on FN1, ZEB1, ZEB2, KRT7, KRT8 and KRT18 mRNAs. Only those genes predicted to be targeted by miR-200 family members were observed to be down regulated in cells in which they were ectopically overexpressed. A: miRanda (mirSVR ≤ -0.2), B: miRDB, C: TargetScan (conserved miRNA sites).

	miR-200b			**miR-200c**			**miR-429**			**miR-200a**			**miR-141**		
	A	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**	**A**	**B**	**C**
FN1	2	2	1	2	2	1	2	2	1	0	0	0	0	0	0
ZEB1	5	6	5	5	6	5	5	6	5	3	3	3	3	3	3
ZEB2	6	5	6	6	5	6	6	5	6	3	4	2	3	4	2
KRT7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
KRT8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
KRT18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

HEY cells transfected by miR-200 family members display significant variation in sensitivity to cisplatin associated with variation within the non-seed region of individual miRNAs

Previous studies indicate that EMT is often associated with decreased sensitivity of a variety of cancer cells to chemotherapy (Shah, Summy et al. 2007, Adam, Zhong et al. 2009, Arumugam, Ramachandran et al. 2009). Consistent with this observation, ovarian cancer cells undergoing EMT have been reported to display a decreased sensitivity to platinum-based drugs, a frequently employed first line therapeutic in the treatment of ovarian cancer (Haslehurst, Koti et al. 2012, Marchini, Fruscio et al. 2013). Since EMT is reported to decrease the sensitivity of OC epithelial cells to platinum-based

drugs, we were interested in determining if miR-induced MET of mesenchymal-like OC cells would induce an opposite effect, i.e., be associated with an increased sensitivity of OC cells to platinum-based drugs. To explore this possibility, we monitored the drug susceptibility (IC50) of HEY cells to cisplatin after miRNA-induced MET relative to controls.

The results presented in Figure 2.5 demonstrate that ectopic over expression of all members of the miR-200 family results in a significant increase in cisplatin sensitivity relative to negative control. While notable variation was observed among cells transfected by different members of the miR-200 family, in most cases this variation was not statistically significant. Cells transfected with miR-200b were significantly more sensitive to cisplatin than those transfected with miR-429 ($p < 0.05$). Interestingly, these two miRNAs share identical seed regions suggesting that non-seed sequences may be of significance in miR-200b induced drug sensitivity.

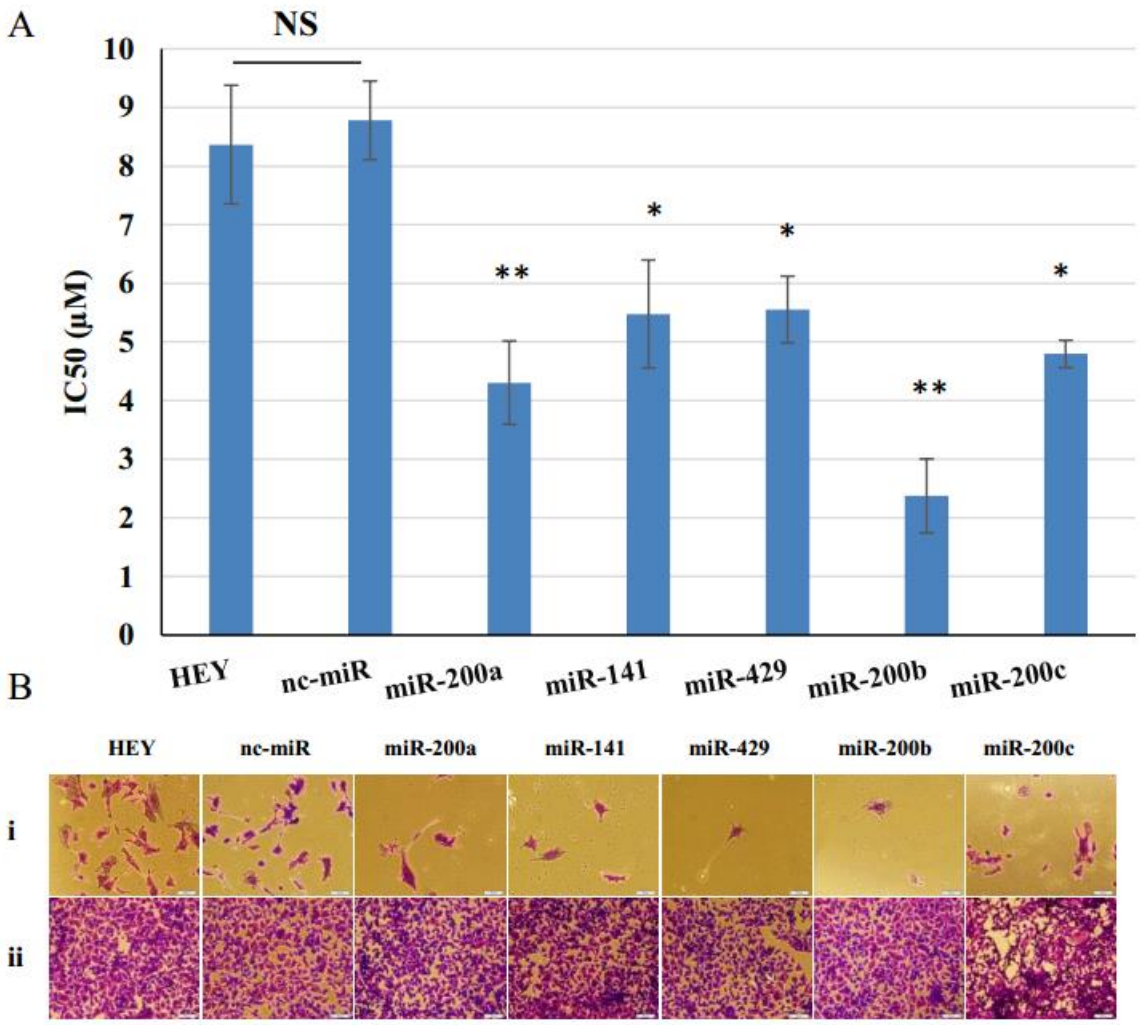


Figure 2.5. Effect of overexpression of members of miR-200 family in HEY cells on cisplatin sensitivity. (A) IC50 values are presented as mean ± SEM of at least 3 independent experiments with 3 replicated wells per drug concentration. Statistical analysis is performed using ANOVA and Tukey's multiple comparison test as a post-hoc test and significant differences from the negative control (nc-miR) group are presented, *P ≤0.05, **P ≤0.005. (B) Qualitative evaluation of cell viability in presence of (i) 20 µM and (ii) 0 µM cisplatin in HEY cells alone or transfected with different members of miR-200 family or nc-miR (crystal violet staining). Scale bars represent 100 µm.

Discussion

It is widely acknowledged that the metastatic spread of cancer cells is responsible for most cancer related deaths (Mehlen and Puisieux 2006, Bacac and Stamenkovic 2008). This is particularly true for ovarian cancer where the prospect of favorable

outcome drops precipitously once cancer cells migrate beyond the confines of the primary tumor (Howlader N). Since the EMT/MET process is believed to play a central role in the metastasis of many cancers (Thiery 2002) including OC (Vergara, Merlot et al. 2010, Lili, Matyunina et al. 2013), considerable effort is being focused on the discovery and development of reagents that may intervene in this process for therapeutic benefit (Monteiro and Fodde 2010).

We previously demonstrated that ectopic over expression of miR-429, a member of the miR-200 family of microRNAs, is sufficient to convert highly metastatic OC mesenchymal-like cells to epithelial phenotype with a concomitant reduction in invasive and migratory potentials (Chen, Wang et al. 2011). Prompted by these initial results, we were interested in determining if the MET-inducing properties of miR-429 extended to other OC mesenchymal-like cell lines and if other members of the miR-200 family were also capable of exerting these potentially anti-metastatic effects. Finally, since previous studies have shown that ovarian cancer cells induced to undergo EMT display a significantly decreased sensitivity to platinum-based drugs (Haslehurst, Koti et al. 2012, Marchini, Fruscio et al. 2013), we were interested to determine if miRNA-induced reversal of the process (i.e., MET) might increase sensitivity to these drugs thereby demonstrating an additional potential therapeutic benefit of these small regulatory RNAs.

Consistent with our earlier studies, we found that ectopic overexpression of miR-429 in two additional OC mesenchymal-like cell lines (SKOV-3 and HEY A8) results in morphological and molecular changes characteristic of MET. Ectopic over expression of other members of the miR-200 family in mesenchymal-like HEY cells also induced morphological changes characteristic of MET but underlying molecular changes were

found to be variable and attributable to sequence variation within the seed region of individual family elements. Finally, we tested the sensitivity of HEY cells to cisplatin after transfection with members of the miR-200 families relative to negative controls. The results demonstrate that although miR-200 family induced MET is generally correlated with a significant increase in sensitivity to cisplatin, significant variation exists among individual family members. In this case, the variability was associated with sequence variation mapping to the non-seed region of individual family members.

Conclusions

Collectively our results are consistent with earlier findings from our lab (Chen, Wang et al. 2011) and others (Gregory, Bert et al. 2008, Park, Gaur et al. 2008) indicating that members of the miR-200 family are involved in EMT/MET and ovarian cancer metastasis. Our results also indicate that significant variation exists among family members in the regulation of molecular processes underlying specific features of the EMT/MET process and that this variation is associated with sequence variation mapping to both the seed and non-seed region of individual family members. Our results generally support the notion that exogenous modulations in the expression of miRNAs involved in EMT may serve as the basis of important new strategies in the treatment of ovarian and other types of cancer (Xia and Hui 2012).

Methods

Cell culture and miRNA transfection

HEY and HEY A8 ovarian cancer cell lines were provided by Gordon B. Mills (MD Anderson Cancer Center, Houston, TX). SKOV-3 ovarian cancer cells were

obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured in RPMI 1640 (Mediatech, Manassas, VA) supplemented with 10% FBS (Fetal Bovine Serum; Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotic-antimycotic solution (Mediatech-Cellgro, Manassas, VA). For miRNA transfections, 6×10^4 cells were seeded per well in 24-well plates. Cells at exponential phase of growth were transfected with 30 nM miRNA purchased as Pre-miR miRNA Precursors (Ambion, Austin, TX) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and according to the manufacturer's instructions. Cells were allowed to grow for 48 hours before RNA isolation. Ambion Pre-miR miRNA Precursor Negative Control was used as a negative control (nc-miR).

Image capture and morphological assessment

Morphological changes were monitored using an Olympus IX51 microscope (Olympus Optical, Melville, NY). The effect of treatment on cell morphology was objectively measured using CellProfiler cell-imaging software (2.1.0) (Carpenter, Jones et al. 2006).

Quantitative reverse transcription real-time PCR (qRT-PCR)

Total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Valencia, CA). RNA concentrations were measured using a NanoDrop 1000 Spectrophotometer V3.2 (NanoDrop, Wilmington, DE). Highly pure RNA samples (A260/A280 between 2.0 and 2.1) were converted into first-strand cDNA with the Superscript III First-strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time PCR analyses were performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) on the CFX96 real-time PCR system (Bio-Rad, Hercules, CA).

The primer sequences employed are as follows: KRT7 (keratin 7): forward 5′-GGACATCGAGATCGCCACCT-3′ and reverse 5′- ACCGCCACTGCTACTGCCA-3′; KRT8 (keratin 8): forward 5′- CCGTGGTTGTGAAGAAGATCG-3′ and reverse 5′-GCTGTTCACTTGGGCAGGAC-3′; KRT18 (keratin 18): forward 5′-TGAGACGTACAGTCCAGTCCTT-3′ and reverse 5′-GCTCCATCTGTAGGGCGTAG-3′; GAPDH: forward 5′-TGCACCACCAACTGCTTAGC -3′ and reverse 5′- GGCATGGACTGTGGTCATGA -3′. The primer sequences for mesenchymal biomarker genes were described previously (Chen, Wang et al. 2011). The threshold cycle and ΔΔCt method was used for calculating the relative amount of the target RNA. Expression values were normalized using GAPDH as a reference gene.

Immunostaining

5×10^3 cells were cultured on eight well chamber slides. Cells at exponential phase of growth were transfected as indicated above. 48 hours after transfection, media was removed and cells were fixed in 10% neutral buffered formalin for 15 min. Cells were then washed, permeabilized with 0.5% Triton X-100 for 5 min, washed again and blocked with 5% BSA (Bovine Serum Albumin) in PBS for 1 hour. The slides were incubated with mouse monoclonal primary antibodies against CDH1 (E-cadherin) and FN1 (Fibronectin, 1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in 5% BSA for 1 hour at room temperature. After washing, the slides were then incubated with Alexa Fluor 488 Rabbit Anti-Mouse secondary antibody (1:500, Molecular Probes, Inc., Eugene, OR) for 1 hour at room temperature. After a wash with PBS, counterstaining was performed using DAPI (4′,6-diamidino-2-phenylindole, 1:2000) in PBS for 30 min. Cells

were then mounted and expression status of protein biomarkers was assessed using the Zeiss confocal microscope system (Carl Zeiss, Jena, Germany).

Cisplatin sensitivity

Aliquots of cells were seeded in 96-well plates and treated with nine concentrations of cisplatin ranging from 0.1-50 μM. After 72 hours, TOX-8 reagent (Resazurin based in vitro toxicology assay kit, Sigma-Aldrich, St Louis, MO) was added to the wells and fluorescence was measured ($\lambda ex = 560$ nm, $\lambda em = 590$ nm). Outlier values were removed using Grubbs' test. The ratio of the background-subtracted fluorescence intensities of drug treated to untreated cultures was calculated in percentages across all concentrations. IC50 values were determined by non-linear regression of log-transformed data using a normalized response-variable slope model with GraphPad Prism v.6 (GraphPad Software Inc., La Jolla, CA).

Statistical analysis

Statistical significance of differences in qRT-PCR experiments between experimental and control samples was determined using a two-tailed Student's t-test. Significance of differences in eccentricity profiles of experimental and control samples was evaluated using Mann-Whitney U test. Statistical significance of differences in mean IC50 values among miRNA transfections was tested using analysis of variance (ANOVA) and Tukey's multiple comparison test as a post-hoc test. All experiments were performed using at least three biological replicates.

CHAPTER 3

FUNCTIONAL AND EVOLUTIONARY SIGNIFICANCE OF HUMAN MICRORNA SEED REGION MUTATIONS

Preliminary comments

The contents of this chapter are published in PLoS ONE under the title "Functional and Evolutionary Significance of Human MicroRNA Seed Region Mutations" with me listed as a first coauthor. Dr. Chris Hill and I are co-first authors on this paper. Dr. Hill carried out all of the computational analyses on the relationship between miRNA seed variation and predicted target overlaps. I designed and conducted all of the wet lab experiments including cell culturing, transfection, RNA extractions. I collaborated with Dr. Lilya Matyunina in carrying out the microarray experiments and with Chris Hill on analysis of the microarray data.

Abstract

MicroRNAs have emerged in recent years as important regulators of cell function in both normal and diseased cells. MiRNAs coordinately regulate large suites of target genes by mRNA degradation and/or translational inhibition. The mRNA target specificities of miRNAs in animals are primarily encoded within a 7 nt "seed region" mapping to positions 2–8 at the molecule's 5′ end. We here combine computational analyses with experimental studies to explore the functional significance of sequence variation within the seed region of human miRNAs. The results indicate that a substitution of even a single nucleotide within the seed region changes the spectrum of mRNA targets by >50%. The high functional cost of even single nucleotide changes

within seed regions is consistent with their high sequence conservation among miRNA families both within and between species and suggests processes that may underlie the evolution of miRNA regulatory control.

Introduction

MiRNAs are small nucleotide RNA molecules that play important regulatory roles in cell function (Nazarov, Reinsbach et al. 2013), embryonic development (Alvarez-Garcia and Miska 2005) and the onset and progression of a variety of diseases (Ha 2011), including cancer (Nana-Sinkam and Croce 2010). Like siRNAs and other small regulatory RNAs, miRNAs regulate their target genes by mRNA degradation and/or translational inhibition (Bartel 2004). However, unlike siRNAs that target one or a few genes, individual miRNAs have evolved the ability to coordinately regulate large suites of target genes, many of which may encode coordinated cellular functions (Bartel 2004, Shahab, Matyunina et al. 2011). The mRNA target specificities of miRNAs in animals are primarily encoded within a 7 nt "seed region" mapping to positions 2–8 at the molecule's 5′ end (Bartel 2009, Wang 2014).The importance of this 7 nt sequence to miRNA function is evidenced by the fact that the seed region sequence of many miRNA families is highly conserved both within and between species (Wheeler, Heimberg et al. 2009). Mature single-stranded miRNAs bound to the RNA-induced silencing complex (RISC) recognize their regulatory targets by Watson-Crick base pairing to compatible sequences (usually in 3′ un-translated regions or 3′ UTRs) in target mRNAs.

It is estimated that there are >1000 sequentially distinct miRNAs in the human genome, each being present in a few to hundreds of copies (Kozomara and Griffiths-Jones 2014). We focused on 249 human miRNAs previously shown to be sequentially

conserved across mammalian species (Lewis, Shih et al. 2003). In this study, we combine computational analyses with experimental studies to explore the functional significance of sequence variation within the seed region of human miRNAs. Our computational analyses predict that as few as one nucleotide change within this 7-nt seed region will alter the spectrum of targeted mRNAs by >60–70%. Further nucleotide substitutions are predicted to have little to no additional effect. Ectopic over expression of synthetic miRNAs with variable seed regions (miR-429, miR-141 and miR-205) but with identical (miR-429) non-seed regions were conducted to experimentally evaluate the consequence of differences in seed region on patterns of gene expression. The experimental results again indicate that as few as one nucleotide change within seed regions results in >50% alteration in the spectrum of mRNAs directly or indirectly regulated by the over expressed miRNA. Further nucleotide differences (5 nucleotide differences) within the seed region were found to have no additional effect. The high functional cost of even a single nucleotide change within the seed region of human miRNAs is consistent with the rigidly conserved seed sequence identity among miRNA families both within and between species (Wheeler, Heimberg et al. 2009) and suggests possible mechanisms underlying the evolution of miRNA regulatory control.

Results and Discussion

The functional consequence or "cost" of seed region nucleotide changes involves the loss of regulatory control over previously targeted mRNAs and/or the acquisition of novel regulatory control over previously untargeted mRNAs. To systematically explore this phenomenon computationally, we first determined the number of nucleotide changes needed to transform one seed region into another (Hamming distance) for each of the 249

miRNAs analyzed in this study. We then calculated the percent overlap (cosine similarity) of predicted targets for all pairs of miRNAs having identical seeds. For example, the percent target gene overlap for miR-25 and miR-32 (both having the seed sequence: 5′AUUGCAC) predicted by miRanda-mirSVR is 81% (Fig. 3.1a). The 19% (100%–81%) divergence in non-overlapping targets is attributable to sequence variation mapping to the non-seed regions (see Figure A.1 for an additional example). The median percent overlap between all pairs of conserved miRNAs with identical seeds is 88% (Figure 3.1c; Table A.1; n = 144), with an average of 12% divergence in non-overlapping genes attributable to variation in non-seed regions.

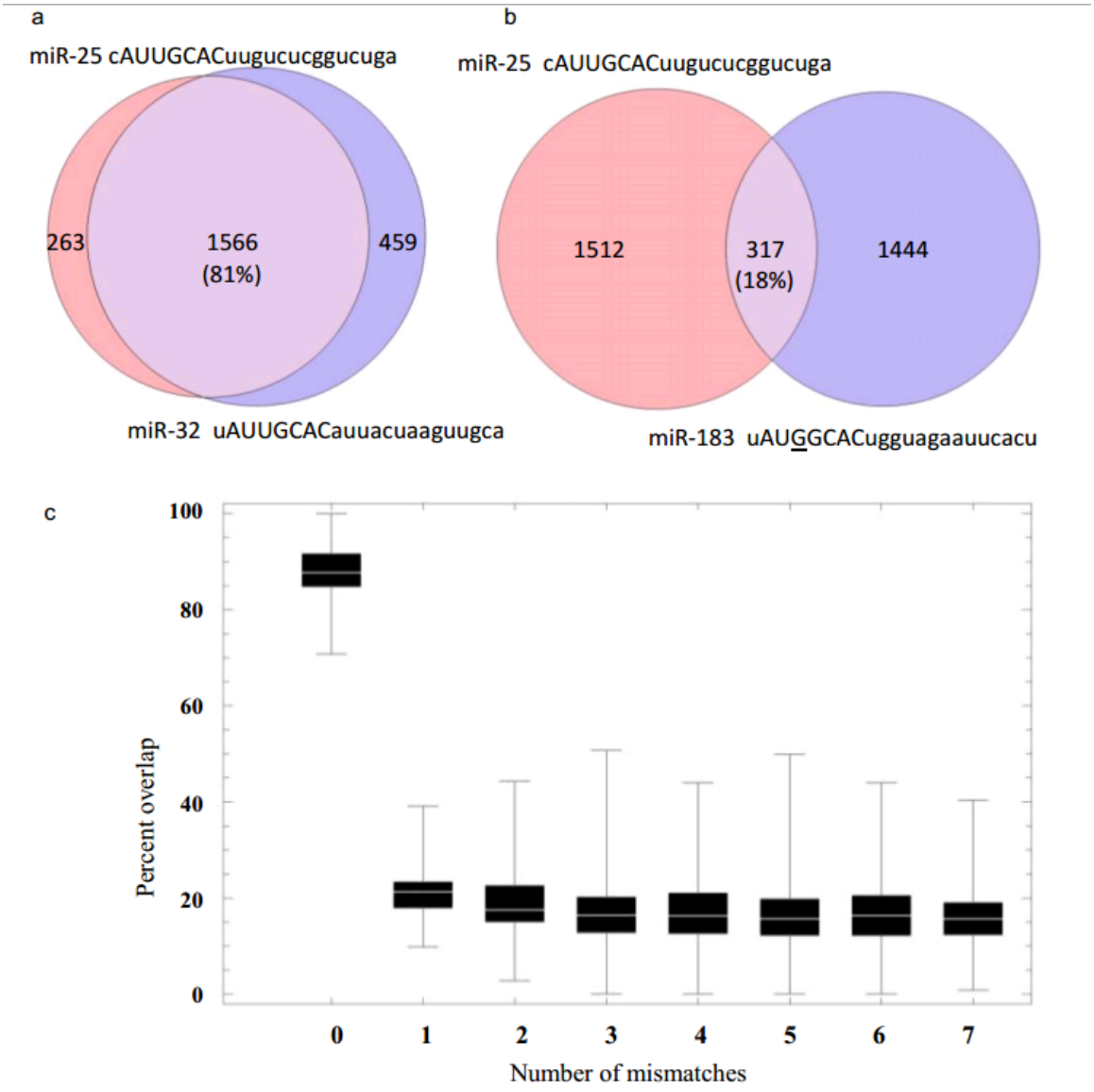


Figure 3.1. A single nucleotide difference in seed region of miRNAs is associated with a major change in regulated target genes. a) miR-25 and miR-32, two miRNAs with identical seed regions (upper-case letters), have 81% overlap in their predicted target genes; b) miR-25 and miR-183, two miRNAs with a single nucleotide difference within their respective seed regions have only 18% overlap in their predicted target genes; c) The overlap of predicted targets for all 249 pairs of conserved miRNAs grouped by the number of mismatches in their respective seed regions. The computed percentage overlaps (cosine similarity) are presented as box and whisker plots [the bottom and top of each box represent the first and third quartiles of variation while the band inside the box represents the median (second quartile) value; the "whiskers" represent variability outside the upper and lower quartiles].

We next independently computed the average percent overlap of predicted mRNA targets for pairs of miRNAs having seeds that differ by 1 to 7 nucleotides. The results (Figures 3.1b, 3.1c, A.1b; Table A.2) indicate that even a single nucleotide mismatch in the seed regions of two miRNAs is computationally predicted to reduce the percent overlap (and increase the percent of non-overlap) among their respective targeted mRNAs by >70%. The generality of these computational predictions was corroborated independently by conducting the same analyses using two additional target prediction algorithms, TargetScan and PicTar (Figures A.2a, A.2b). Thus, the computational studies consistently predict that as few as one nucleotide substitution within the seed region of miRNAs will be associated with significant functional cost and that further changes will have little or no additional cost.

While informative in their own right, functional predictions based on target prediction algorithms alone are often inaccurate in vivo because they ignore the myriad of indirect regulatory effects induced by miRNAs (Shahab, Matyunina et al. 2011, Lu and Clark 2012, Hill, Matyunina et al. 2014). In an effort to experimentally explore the functional cost of nucleotide variation within miRNA seed regions, we selected for analysis members of the miR-200 family of human miRNAs that differ by a single nucleotide in the seed region (miR-429 vs. miR-141 differ by 1 nucleotide at position 4) or differ by multiple nucleotides (miR-429 and miR-205 differ by 5 nucleotides at positions 2,3,5,7 and 8) within their respective seed regions (Figure 3.2). In order to avoid confounding effects attributable to variation within non-seed regions and to focus on the significance of seed region variation, synthetic derivatives of these naturally occurring miRNAs were constructed to have identical miR-429 non-seed regions (Figure

3.2). In addition, to explore the possible significance of variability in the position of single nucleotide differences within seed regions, we constructed an additional miR-429 variant with a single nucleotide substitution at seed region position 2 (M12, Figure 3.2). Each of these miRNAs were independently transfected into the well-characterized HEY ovarian cancer cell line (Buick, Pullano et al. 1985) and after 48 hrs RNA was extracted and subjected to gene expression analysis (Affymetrix, U133) as previously described (Shahab, Matyunina et al. 2012, Jabbari, Reavis et al. 2014) (Tables A.3-A.6 for detailed results).

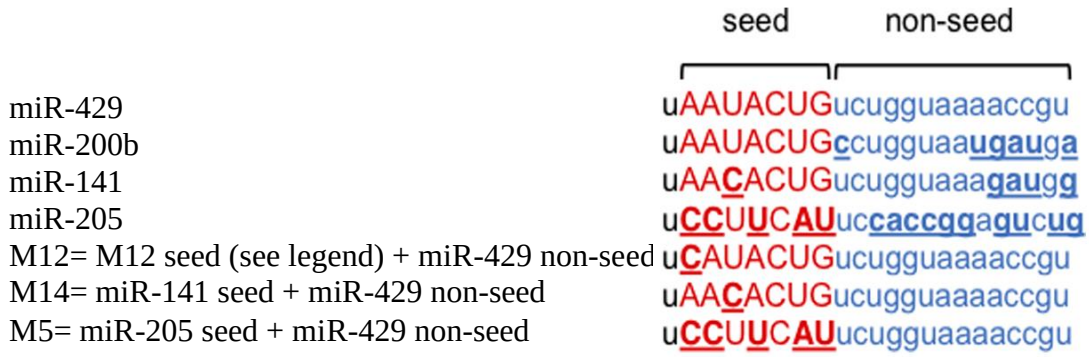


Figure 3.2. Sequence alignments of miR-429, miR-200b, miR-141, miR-205, M12, M14 and M5. Seed and non-seed regions are colored in red and blue, respectively. Differences in seed and non-seed regions of miRNAs relative to miR-429 are underlined. M12, 14 and M5 all have identical non-seed regions with miR-429. M12 differs in seed region sequence from miR-429 by one nt at position 2. The seed region sequence of M14 is identical to that of miR-141 and differs from the seed of miR-429 by one nucleotide at position 4. M5 differs in seed region sequence from miR-429 by 5 nucleotides.

As mentioned above, the functional cost associated with nucleotide changes within seed regions is reflective of the loss of regulatory control over previously targeted mRNAs and/or the acquisition of novel regulatory control over previously untargeted mRNAs. We experimentally estimated these parameters by comparing all significantly

differentially expressed genes in cells transfected by miRNAs with seeds differing by 0 (miR-429 vs miR-429), 1 (miR-429 vs M12 and miR-429 vs M14) or 5 (miR-429 vs M5) nucleotides. Presented in Fig. 3.3c is the observed percent overlap of all significantly differentially expressed genes among 3 replicate transfections with miR-429. The high similarity in percent overlap among these replicate miR-429 transfections is indicative of the low experimental error associated with the technique. Figs. 3.3a and 3.3b present the observed percent overlap in significantly differentiated genes in cells transfected with miR-429 vs cells transfected by miRNAs differing from miR-429 by a single nucleotide within their respective seed regions. The differences are highly significant ($\chi 2 = 1511$; $p<0.0001$) and consistent with the prediction that a substantial functional cost is associated with even a single nucleotide change within miRNA seed regions. Differences in overlap between the miR-429 vs M12 and miR-429 vs M14 comparisons were not found to differ significantly ($\chi 2 = 1.95$; $p<0.16$) indicating that, in this experimental context, position of the single nucleotide change is not significant with respect to functional cost.

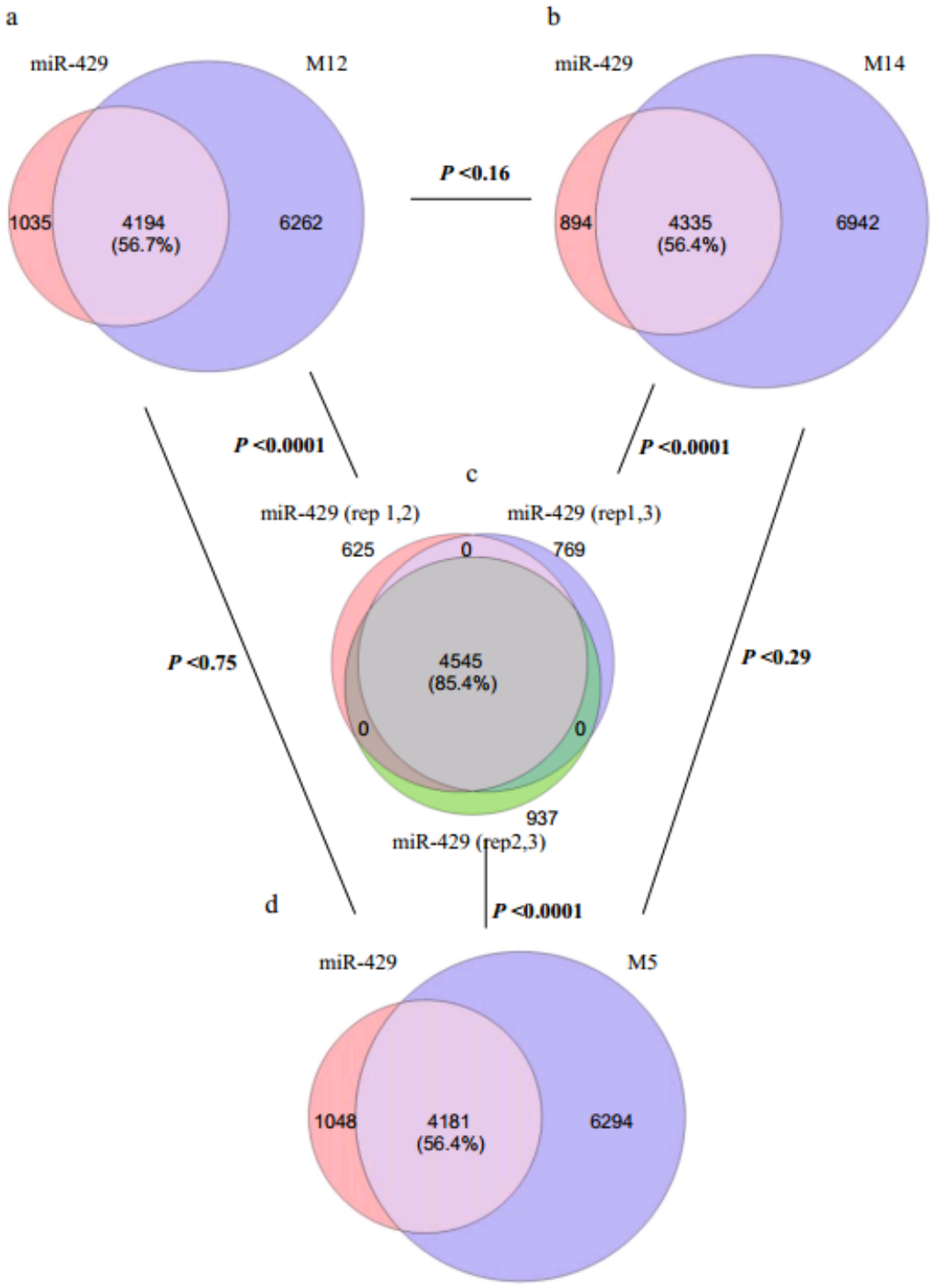


Figure 3.3. Overlap among differentially expressed genes in HEY cells after ectopic expression of miRNAs with different seed region sequences. An overlap of 85% in differentially expressed genes was observed among 3 replicate experiments in which miR-429 was ectopically expressed in HEY cells (c). A single nucleotide change in the seed region of miR-429 (M12, M14) resulted is a significant reduction (56% vs 85%) in

the overlap among differentially expressed genes (a, b). Differences in the position of this single nucleotide difference (M12, position 2 vs M14, position 4) did not have a significant effect on degree of overlap (a vs.b). Five nucleotide differences in the seed region of miR-429 (M5) also resulted in a significant reduction in overlap (56% vs. 85%) among differentially expressed genes (d vs. c) but not significantly different from miRNAs differing by only 1 nucleotide difference from miR-429 (d vs. a; d vs b).

Changes in the percent overlap of significantly differentially expressed genes between cells transfected by miR-429 vs those transfected by the miRNA differing at 5 nucleotide positions within the seed region (Figure 3.3d) were also highly significant ($\chi 2 = 1535$; $p<0.0001$) but not significantly different from the changes induced by miRNAs with only a single nucleotide difference from miR-429 ($\chi 2 = 0.1$; $p<0.75$).

Collectively, the above results indicate that even a single nucleotide substitution within the seed regions of miRNAs is associated with substantial functional cost and suggests an evolutionary model whereby strong stabilizing selection is maintaining rigid conservation of miRNA seed sequences both within and between species. Individual target genes, on the other hand, may acquire and/or lose miRNA regulatory control(s) through even single nucleotide substitutions in miRNA target sequences complimentary to miRNA seeds (typically within 3′ UTRs) (Figure 3.4). Any functional consequence of such mutations would be incurred on the individual gene level rather than on the multi-gene level associated with miRNA seed region mutations. This implies that although seed regions may be highly conserved both within and between species due to strong stabilizing selection, the spectrum of genes regulated by these sequentially conserved miRNAs may be expected, on average, to vary significantly, especially between more distantly related species where there has been ample time/opportunity for individual

genes to acquire variation in their target sequence(s) and to re-associate themselves with other, presumably adaptive, miRNA regulatory controls (directional selection).

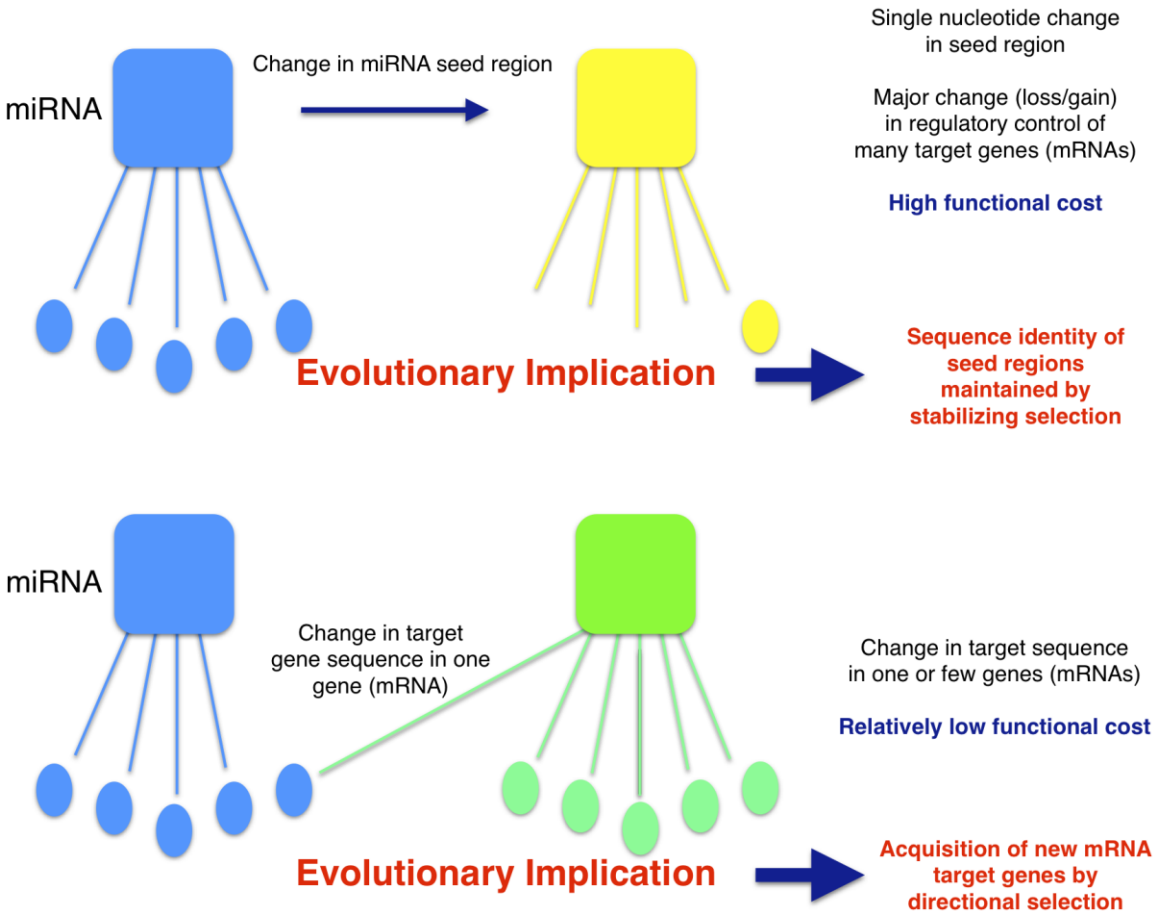


Figure 3.4. The high functional cost of even single nucleotide changes in miRNA seed regions implies a mechanism of miRNA regulatory evolution. If a miRNA (blue square) changes a single nt within its seed region (yellow square), a significant fraction of its target genes (blue and yellow ovals) will change resulting in high functional cost. As a result, strong stabilizing selection is expected to maintain miRNA seed region sequence identity among miRNA families within and between species. In contrast, individual genes may change miRNA regulatory control(s) (blue to green squares) by as little as a single nucleotide change in their target sequences (blue to gene ovals) with relatively low functional cost. Thus, evolutionary changes in miRNA regulatory control are likely to be driven primarily by random and/or adaptive (directional selection) changes in target gene sequences.

As an initial test of this prediction, we selected two miRNAs (miR-429 and miR-200b) that have identical seed regions in both humans and mice (Figure 3.5a). We employed the miRanda-mirSVR algorithm to predict the respective orthologous mRNA targets of these two miRNAs (human: hsa-miR-429, hsa-miR-200b; mouse: mmu-miR-429, mmu-miR-200b) in both species. As shown in Figure 3.5b, the percent overlap between the predicted gene targets of these two miRNAs (intra-specific) is >90% (mouse: 93.3%; humans: 91.8%) in both species. However, despite the fact that the human and mouse miRNAs share sequentially identical seed regions, they display <40% overlap among their respective target genes/mRNAs in the non-native species (inter-specific) (Figure 3.5b). To determine if these differences are representative of other sequentially conserved miRNAs, we computed the percent overlap of genes targeted by the 249 miRNAs sequentially conserved in mouse and humans. The results confirm that the average overlap between targeted genes in mouse and humans is <30% (Figure 3.5c). This dichotomy is well below the false positive values expected given the high stringency cut-off values used in our predictions (mirSVR score <−0.2) (Betel, Koppal et al. 2010). These results are consistent with the hypothesis that while miRNA seed regions may be selectively conserved across species, target genes maintain relative flexibility to acquire and/or lose miRNA regulatory controls by even single nucleotide changes within their respective miRNA target sequences (typically within 3′ UTRs).

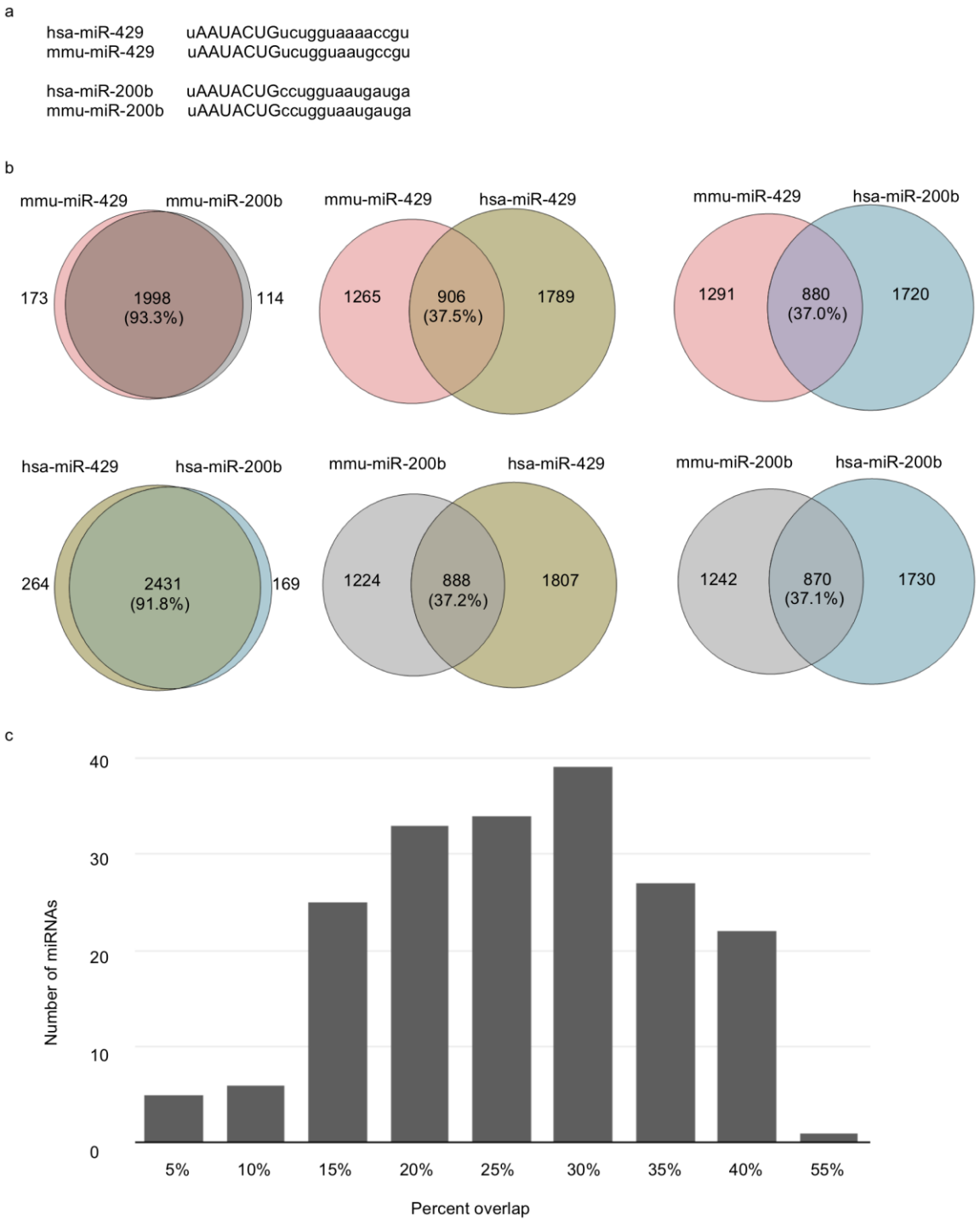


Figure 3.5. Mouse and human miRNAs share sequentially identical seed regions but regulate highly divergent groups of target genes. a) Sequence alignment between human (has-miR-429/200b) and mouse (mmu-miR-429/200b) miR-429 and miR-200b miRNAs. Despite the substantial evolutionary distances between these two species, the respective seed regions are sequentially identical indicative of strong stabilizing selection. b) Venn diagrams showing the % overlap in predicted miR-429 and miR-200b target genes between human and mouse orthologs. c) The average percent overlap of

genes predicted to be targeted by all 249 sequentially conserved mouse and human miRNAs is <30%. The results are consistent with a model of miRNA regulatory evolution whereby miRNA seed region sequences are selectively conserved while target genes may rapidly re-position themselves under novel miRNA regulatory control(s).

Collectively, our findings support an evolutionary model whereby miRNAs initially evolve to regulate large suites of target genes. Thereafter, the sequential integrity of miRNA seed regions is maintained by strong stabilizing selection due to the high functional cost of even a single nucleotide mutation within miRNAs. In contrast, nucleotide mutations in the target sequences of individual genes, being, on average, of substantially lower functional cost, allow for a relatively rapid repositioning of miRNA-target gene associations. Indeed, a variety of scenarios might arise to buffer the possible negative effects of target sequence mutations in regulated genes. For example, duplication of specific target sequences within regulated genes could serve to mask the impact of the sudden loss of existing miRNA regulatory controls while still permitting genes to explore the potential adaptive benefits of acquiring new miRNA regulatory controls.

Materials and Methods

Computational predictions of miRNA target and overlap

To determine the mRNA targets of the 249 conserved miRNAs, we utilized three online target prediction programs, miRanda-mirSVR (Betel, Koppal et al. 2010), TargetScan (Friedman, Farh et al. 2009) and PicTar (Krek, Grun et al. 2005). The miRanda predictions are driven by mirSVR, an application that uses machine learning to evaluate and score the importance of various features from miRNAs and their putative

target sites. Predicted miRNA targets were filtered for targets with a mirSVR score less than −0.2 to minimize false positives. Corroborative predictions were carried out using TargetScan and PicTar.

We determined the distance between two sequences by calculating the Hamming distance (Hamming 1950). That distance is calculated by counting the number of nucleotide changes needed to transform one seed sequence into another. Overlap between the predicted miRNA targets was determined using cosine similarity (Tan 2005), calculated by dividing the total number of overlapping genes by the square root of the product of the number of genes targeted by each miRNA. Taking the square root of the number of predicted targets reduces the influence of miRNAs with abnormally large numbers of targets and simultaneously normalizes the result, generating a score between 0 and 1. The significance of the difference in overlap of differentially expressed genes between two pairs of miRNAs was calculated using the chi-square test of association; the overlap and cost (non-overlap) for each pair were compared.

Cell culture and transfections

HEY (Buick, Pullano et al. 1985) ovarian cancer cells, provided by Gordon B. Mills (MD Anderson Cancer Center, Houston, TX), were cultured in RPMI-1640 (Mediatech, Manassas, VA) with 10% Fetal Bovine Serum (FBS, Atlanta Biologicals, GA) and 1% antibiotic-antimycotic solution (Mediatech-Cellgro, Manassas, VA), and incubated at 37°C and 5% CO2. The transfection protocol was as described previously (Jabbari, Reavis et al. 2014). Briefly, triplicate wells of exponentially growing cells were transfected with miR-429, and the custom designed miRNAs M12, M14 and M5 purchased as Pre-miR miRNA Precursors (Ambion, Austin, TX). Transfections were

performed using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Ambion Pre-miR miRNA Precursor Negative Control was used as a negative control.

RNA isolation and whole genome microarray

RNA was extracted from transfected cells using the RNeasy Mini RNA isolation kit (QIAGEN, Valencia, CA). Microarray experiments were performed as previously described (Shahab, Matyunina et al. 2012). Briefly, RNA samples with high integrity were converted to cDNA and amplified with Applause 3′-Amp System (NuGen, San Carlos, CA). The cDNA was fragmented and Biotin labeled using the Encode Biotin Module (NuGen). Labeled cDNA was then hybridized to Affymetrix HG-U133 Plus 2.0 arrays and analyzed with GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA).

Microarray data analysis

To determine differentially expressed genes in triplicates of experimental miRNA and negative control treated cells, the following procedure was followed. Quality control was first assessed using all raw CEL files as implemented in Array Analysis (Eijssen, Jaillard et al. 2013). GC Robust Multi-array Average (GCRMA) normalization was performed using all CEL files that passed quality control (n = 3 per experimental group after quality assessment). For GCRMA normalization, each experimental miRNA group was compared independently to the negative control group. Next, probe set filtering was performed as follows: present/absent calls were first generated by Microarray Suite 5.0 (MAS5.0) using the Affymetrix Expression Console v1.1. Based on present/absent calls, probe sets with less than 50% present calls across all experimental miRNA and negative control samples were removed. In addition, probes and probe sets lacking a gene symbol

annotation were ignored. Based on GCRMA expression signals, calculation of signal-to-noise ratio (SNR = mean/standard deviation) was used to select and keep only the probe sets with the highest SNR for differential expression profiling. GCRMA expression signals for those probe sets were submitted to SAM (Significance Analysis of Microarrays) (Tusher, Tibshirani et al. 2001). Each SAM input file contained triplicates of experimental miRNA and negative control GCRMA expression columns. Differentially expressed genes in each experimental miRNA group were determined compared to the negative control (as a baseline) using the false discovery rate (FDR) <2%. SAM output files included significantly differentially expressed genes (upregulated and down-regulated genes in separate lists). All raw CEL files, MAS5.0 (CHP files) and GCRMA processed files are deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov) SuperSeries number GSE56973.

Orthologous genes

Orthologous genes in human and mouse were identified using the BioMart data-mining tool (Kasprzyk 2011). Briefly, all Homo sapiens genes were selected on BioMart and filtered to remove the genes with no mouse orthologs. The output file included the mouse orthologs for all the resulting genes. These genes were then used for our comparison of miR-429 and miR-200b predictions across human and mouse species.

CHAPTER 4

EVIDENCE FOR FUNCTIONALLY SIGNIFICANT INTERACTIONS BETWEEN SEED REGION AND NON-SEED REGION AMONG MEMBERS OF THE MIR-200 FAMILY OF MICRORNAS

Preliminary comments

Contents of this chapter will soon be submitted for publication. The author contribution to experiments and data analyses are as follows: I designed and conducted all the wet lab experiments including cell culturing, transfection, drug sensitivity assays and PCR gene expression profiling. I collaborated with Dr. Lilya Matyunina in carrying out the microarray experiments.

Abstract

MicroRNAs are small (22bp) non-coding RNAs that regulate large suites of target genes by mRNA degradation and/or translational inhibition. Over-expression of miR-200b in mesenchymal-like ovarian cancer cells significantly increases sensitivity to the chemotherapeutic drug cisplatin. This functionally significant change has been previously associated with sequence variation mapping to the non-seed region on mir-200b. We confirm these earlier findings and report that the miR-200b induced increase in drug sensitivity is correlated with significant changes in patterns of gene expression including the down-regulation of 6 genes previously associated with cisplatin resistance in cancer cells. We further report that substitution of a single nucleotide within the mir-200b seed region results in loss of the ability of miR-200b to impart cisplatin resistance.

Collectively, our results provide evidence for seed region/non-seed interactions in the regulation of miRNA-induced cisplatin resistance in ovarian cancer cells.

Introduction

MicroRNAs (miRNAs) are small (22bp) non-encoding RNAs that play important roles in cell function (Nazarov, Reinsbach et al. 2013) by coordinately regulating large suites of target genes (Bartel 2004, Shahab, Matyunina et al. 2011), Regulation is brought about by miRNA binding to complementary sequences within the mRNAs of target genes that blocks translation and/or leads to mRNA degradation (Bartel 2004). Numerous studies have demonstrated that miRNA binding to target mRNAs is primarily determined by a sequentially conserved region within the miRNA ("seed region") mapping to positions 2-8 within the the 5' end of the miRNAs (Bartel 2009, Wang 2014). Despite the importance of seed region in miRNA function, more recent studies indicate that sequence pairing between seed regions and target mRNAs is not always sufficient for miRNA function (Grimson, Farh et al. 2007). For example, productive 3′ pairing on miRNA nucleotides 13–16 ("3'-supplementary sites") and pairing centered on miRNA nucleotides 13–17 ("3′-compensatory sites"), has been shown to supplement seed region pairing, and/or compensate for a single nucleotide mismatches between miRNAs and their targeted mRNAs mapping to the seed region(Bartel 2009).

We have recently provided preliminary evidence that the non-seed region (position 9-21) of miR-200b plays a significant role in conferring cisplatin sensitivity when ectopically over-expressed in mesenchymal-like, HEY ovarian cancer cells (Jabbari, Reavis et al. 2014). To explore this hypothesis in more detail, we combined the miR-200b non-seed region with a seed region differing from miR-200b by only a single

nucleotide substitution. Combining the miR-200b non-seed region with a heterologous seed region resulted in a the loss of the ability of the chimeric miRNA to confer cisplatin sensitivity when over-expressed in HEY cells and a correlated elevation in genes previously associated with increased cisplatin resistance in a variety of cancer cells. The results indicate that the functional significance of the miR-200b non-seed region is dependent upon the seed region sequence with which it is paired.

Results

The naturally occurring miR-429 and miR-200b microRNAs have identical seed regions but highly divergent non-seed regions (Figure 4.1a). We have recently reported that despite the ability of miR-429 and miR-200b miRNAs to induce Mesenchymal Epithelial Transition (MET) in HEY cells, the two miRNAs confer a significantly different response to cisplatin in these cells. Specifically, miR-200b transfected cells are significantly more sensitive than miR-429 transfected cells (Figure 4.1b) (Jabbari, Reavis et al. 2014). Given that the two miRNAs have identical seed and different non-seed regions, the observed differences in the cisplatin response profiles can be attributed to variation within the non-seed regions.

a

	seed	non-seed
miR-200b	UAAUACUG	CCUGGUAAUGAUGA
miR-429	UAAUACUG	UCUGGUAAAACCGU
M4b	UAACACUG	CCUGGUAAUGAUGA

b

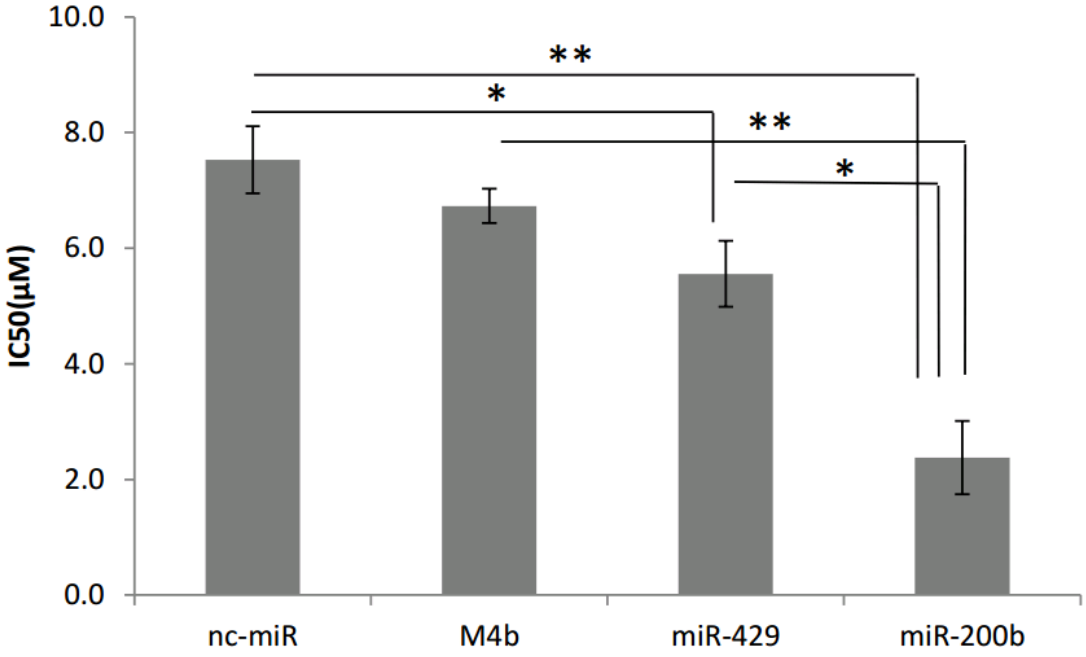


Figure 4.1. Effect of over expression M4b in HEY cells on cisplatin sensitivity. (a) Sequence alignment of mature miR-200b, miR-429 and M4b miRNAs. Nucleotides in red correspond to differences from miR-200b sequence. (b) IC_{50} values are presented as mean ± SEM of at least 3 independent experiments with 3 replicated wells per drug concentration. Statistical analysis was performed using *t*-test. Asterisks represent significant differences from the paired miRNA, * $P \leq 0.05$, ** $P \leq 0.005$.

To test the hypothesis that the miR-200b non-seed region itself is responsible for conferring cisplatin resistance when over-expressed in HEY cells, we produced a synthetic miRNA (M4b) identical in sequence to miR-200b except for a single nucleotide substitution within the seed region (Figure 4.1a). A series of miRNA transfection experiments were carried out with M4b, miR200b and miR-429- a naturally occurring miRNA that shares an identical seed region with miR-200b but is sequentially different

within its non-seed region. MiR-200b was significantly more sensitive to cisplatin than cells over-expressing miR-429. As previously reported (Jabbari, Reavis et al. 2014), this finding associates the observed increase in sensitivity to cisplatin with the non-seed region of miR-200b. However, the cisplatin sensitivity of cells over-expression M4b is indistinguishable from that observed in cells over-expressing miR-429 (Figure 4.1). Thus, the increased sensitivity to cisplatin of cells over-expressing miR-200b and associated with the non-seed region is lost when as little as a single nucleotide substitution is made in the miR-200 seed region sequence. Collectively, our results indicate that the seed and non-seed regions of miR-200b functionally interact with respect to conferring cisplatin sensitivity when over-expressed in HEY cells.

To explore the potential molecular factors leading to the observed variation in cisplatin response, we conducted genome-wide gene expression analysis (Affymetrix hg-u133 plus 2.0 arrays) of hey cells transfected with miR-200b or miR-429. From among the significantly differentially expressed 5229 and 9224 genes in miR-429 and miR-200b transfections respectively, 4368 genes were in common accounting for 62.9% overlap in differentially expressed genes induced by over-expression of these miRNAs. Replicate experiments carried out with miR-429 and miR-200b resulted in overlaps of 85.4% and 88.4% respectively. This variability can be attributed to experimental error. The significantly lower degree of overlap between miR-429 and miR-200b can be attributed to non-seed region effects (Figure 4.2).

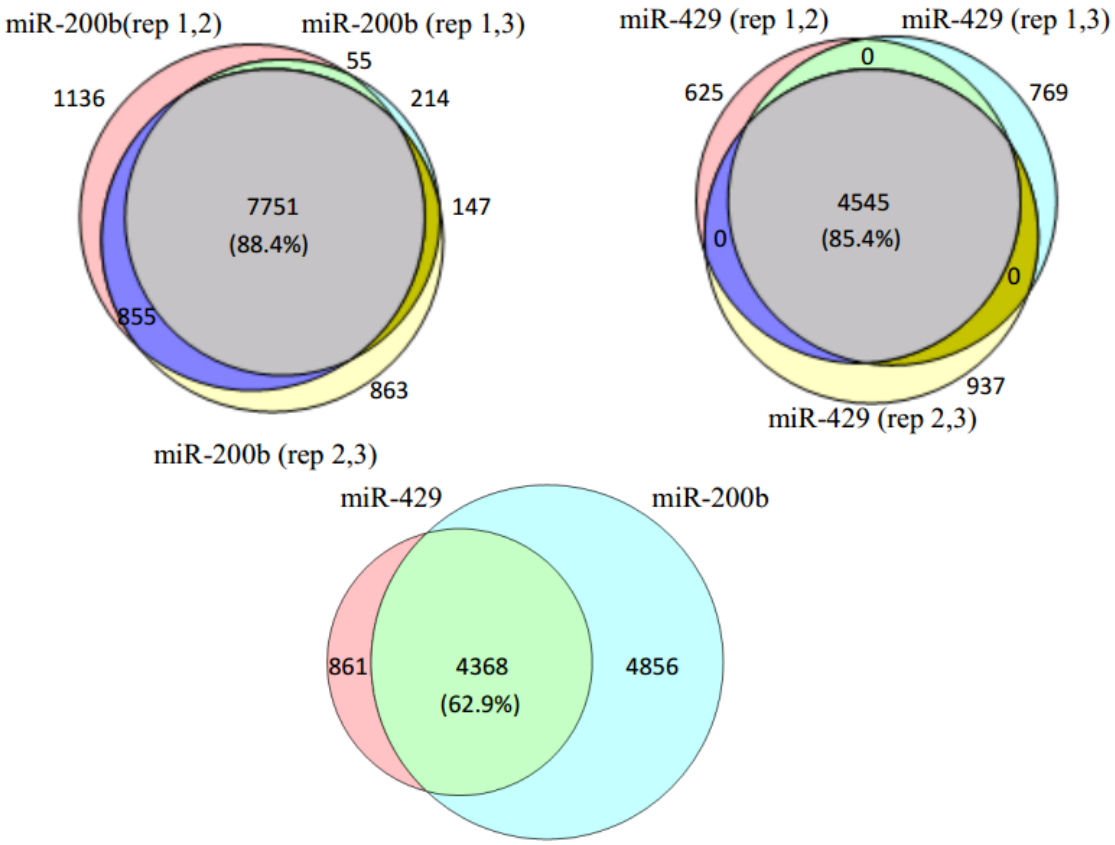


Figure 4.2. Overlap among differentially expressed genes in HEY cells transfected with miRNAs with different non seed regions. An average overlap of 88.4% and 85.4% in differentially expressed genes was observed among any two miR-429 and miR-200b microarray replicates (out of total n= 3) paired with negative control (n=3). Nucleotide differences in the non-seed region of miR-200b (miR-429) resulted is a significant reduction (62.9%) in the overlap among differentially expressed genes.

From among the list of all significantly differentially expressed genes in the microarray experiments, we selected a panel of six genes (Table 4.1) that have been previously associated with cisplatin resistance in a variety of cancers. The microarray analysis indicated that all six of these genes were significantly down-regulated in the miR-200b transfected cells relative to the miR-429 transfected cells consistent with the observed increase in cisplatin sensitivity associated with the miR-200b non-seed region.

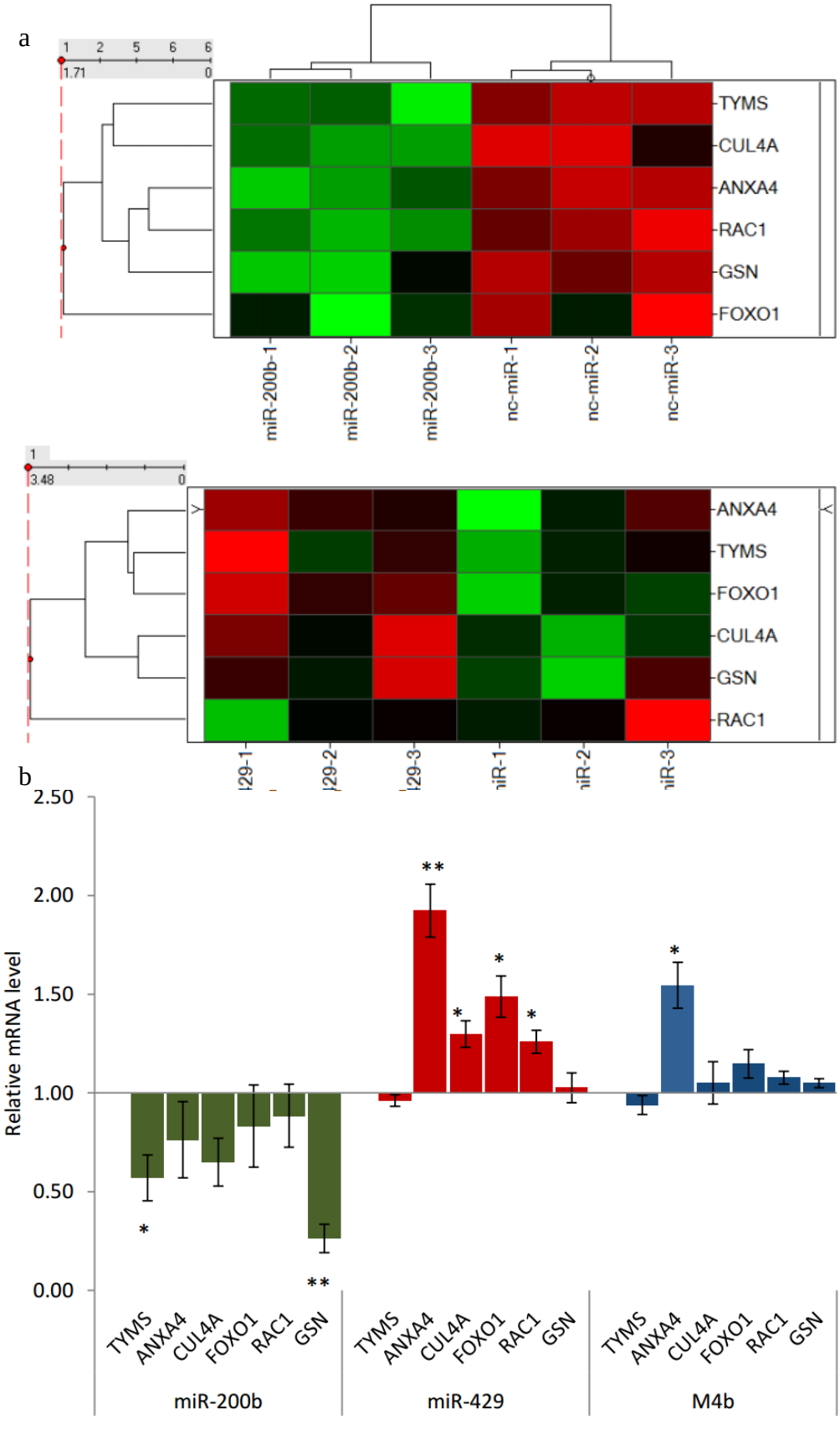
The down-regulation of these genes in HEY cells over-expressing miR-200b relative to cells transfected by miR-429 were confirmed by qRT-PCR (Figure 4.3). Interestingly, the expression pattern of these 6 genes in cells over-expressing M4b was indistinguishable from what was observed in cells over-expressing miR-429 and consistently different from what was observed in cells over-expressing miR-200b. Thus, both the down-regulated pattern of expression of these genes and the observed increase in cisplatin sensitivity associated with the miR-200b non-seed region were lost when a single nucleotide change was made in miR-200b's seed region.

Table 4.1. Representative genes associated with cisplatin resistance that are down regulated in miR-200b transfection and not changed in miR-429 transfection.

Gene	Function/ Association with cisplatin response	Reference
Thymidylate Synthetase (TYMS)	- TYMS catalyzes the reductive methylation of dUMP by CH2H4folate providing the sole de novo source of thymidylate which is essential for DNA replication and repair.	(Carreras and Santi 1995), (Scanlon and Kashani-Sabet 1988)
	- TYMS is important determinant of drug resistance to cisplatin in Human Non–Small-Cell Lung Cancer cells.	(Ko, Tsai et al. 2011)
Annexin A4 (ANXA4)	- An epithelial isoform of a family of soluble cytoplasmic proteins. - Majorly involved in membrane permeability, exocytosis and regulation of ion channels.	(Hill, Kaetzel et al. 2003), (Sohma, Creutz et al. 2001), (Kaetzel, Chan et al. 1994)
	- ANXA4 is overexpressed in ovarian clear cell carcinoma. - Knockdown of its expression in human ovarian clear cell carcinoma cell lines enhances sensitivity to cisplatin and carboplatin.	(Kim, Enomoto et al. 2009), (Morimoto, Serada et al. 2014)
Cullin4A (Cul4A)	- A member of the cullin family of proteins that is in general involved in cell cycle regulation and maintenance of genomic stability.	(Sharma and Nag 2014)
	- Knockdown of its expression leads to inhibition of cancer cell growth and apoptosis. - Knock down of its expression in lung cancer cells increased their sensitivity	(Liu, Lee et al. 2009), (Yang, Hung et al. 2014)

Table 4.1 (continued)

	to cisplatin in vitro.	
Mammalian forkhead transcription factors of the O class (FOXO1)	- Critical regulator of cellular growth and proliferation and are therefore considered to be potential targets for therapeutic strategies against cancer.	(Maiese, Chong et al. 2008)
	- It has been indicated to inhibit cisplatin-induced apoptosis in gastric cancer cells.	(Park, Ko et al. 2014)
Ras-Related C3 Botulinum Toxin Substrate 1 (RAC1)	- It encodes a GTP binding protein belonging to the Rho family of small GTPases, which regulate cellular functions during embryonic development and tumor invasion.	(Fritz, Just et al. 1999)
	- Its down regulation is associated with increased cell sensitivity to cisplatin and cisplatin-induced apoptosis in human cervical epithelial adenocarcinoma cell line	(Xu, Yu et al. 2013)
Gelsolin (GSN)	- A cytoskeleton-associated protein and most cancer cells have significantly lower GSN expression supporting its pro-apoptotic role.	(Sun, Yamamoto et al. 1999), (Kothakota, Azuma et al. 1997), (Tanaka, Müllauer et al. 1995), (Asch, Head et al. 1996), (Zhang, Zhou et al. 1997)
	- Its overexpression inhibits cisplatin induced apoptosis in chemosensitive cells and its down regulation sensitizes cisplatin-resistant cancers.	(Wang, Abedini et al. 2014)


a
1 2 5 6 6
1.71 0
TYMS
CUL4A
ANXA4
RAC1
GSN
FOXO1
miR-200b-1
miR-200b-2
miR-200b-3
nc-miR-1
nc-miR-2
nc-miR-3
1
3.48 0
ANXA4
TYMS
FOXO1
CUL4A
GSN
RAC1
b
Relative mRNA level
2.50
2.00
1.50
1.00
0.50
0.00
TYMS
ANXA4
CUL4A
FOXO1
RAC1
GSN
miR-200b
miR-429
M4b

F**igure 4.3. Expression levels of representative cisplatin resistance genes in miRNA transfected cells are predictive of the cells' response to cisplatin**. Consistent with the higher cisplatin sensitivity in miR-200b transfected cells as compared to miR-429 and M4b transfections, expression levels of representative genes associated with cisplatin are decreased in miR-200b transfection meanwhile not changed or up-regulated in miR-429 and M4b transfections. (a) Heatmaps represent z-score normalized log expression values for the six representative genes in miR-200b, miR-429 tranfections normalized to the nc-miR transfection (Affymetrix HG-U133 Plus 2.0 arrays). (b) Relative expression of the six representative genes in miR-200b, miR-429 and M4b transfected cells using qRT-PCR. Values are normalized to nc-miR transfected cell and represent mean ± SEM of at least three biological replicates each performed in three technical replicates. Asterisks represent significant differences from the negative control group (* $P < 0.05$, ** $P < 0.005$, Student's t-test).

Discussion

MicroRNAs (miRNAs) are endogenous ~22-nucleotide RNAs that play important gene-regulatory roles in animals and plants by pairing to the mRNAs of protein-coding genes to direct their posttranscriptional repression (Bartel 2009). Pairing to the 5' portion of the miRNA, known as seed region and comprised of nucleotides 2–8, appears to be most important for target recognition by vertebrate miRNAs (Lewis, Shih et al. 2003). Most mammalian protein-coding genes are under selection to maintain pairing to the seed of one or more miRNAs (Lewis, Burge et al. , Farh, Grimson et al. 2005) illustrating the broad scope of seed-type regulation.

Despite the demonstrated importance of the seed regions in miRNA targeting, several recent studies indicate that sequence pairing of seed regions with target mRNA sequences is not always sufficient for repression(Grimson, Farh et al. 2007). For example, a recent study by Wang et al., demonstrated that binding stability of the paired nucleotides was a major determinant of miRNA targeting patterns, whether involving seed or non-seed sequences (Wang 2014). Pairing to the 3′ region of the miRNA can supplement seed pairing to enhance target recognition, or it can even compensate for a

single nucleotide bulge or mismatch in the seed region (Bartel 2009). These sites have been designated “3’-supplementary sites” and “3′-compensatory sites”, respectively. In supplementary sites, productive 3′ pairing optimally centers on miRNA nucleotides 13–16 and in “3′-compensatory sites”, the pairing centered on miRNA nucleotides 13–17 extends to at least nine contiguous Watson–Crick pairs (Bartel 2009).

Results reported in this study confirm our earlier evidence for the functional significance of the non-seed region of miR-200b (nucleotides 9-21) in imparting increased cisplatin sensitivity in cells ectopically over-expressing the miRNA. In addition, our results demonstrate that this induced increase in drug sensitivity is correlated with a significant change in global patterns of gene expression including the reduction in levels of expression of six genes previously associated with cisplatin sensitivity in a variety of cancer cells (Table 4.1). Most interesting, was our finding that a single nucleotide change in the seed region of miR-200b is sufficient to lose the functions previously associated with the miRNAs non-seed region. Collectively our results indicate that, at least in some contexts, the functional significance of the non-seed region is dependent upon the seed region with which it is paired.

Material and Methods

Cell culture and miRNA transfection

HEY ovarian cancer cell lines were cultured in RPMI 1640 (Mediatech, Manassas, VA) supplemented with 10% FBS (Fetal Bovine Serum; Atlanta Biologicals, Lawrenceville, GA) and 1% antibiotic-antimycotic solution (Mediatech-Cellgro, Manassas, VA). MiRNA transfections were performed as previously described , Briefly, triplicate 24-well plate wells of cells at exponential phase of growth were transfected

with 30 nM miRNAs purchased as Pre-miR miRNA Precursors (Ambion, Austin, TX) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and according to the manufacturer's instructions. RNA isolation was performed 48 hours post transfection. Ambion Pre-miR miRNA Precursor Negative Control was used as a negative control (nc-miR).

Quantitative reverse transcription real-time PCR (qRT-PCR)

RNeasy mini kit (Qiagen, Valencia, CA) was used to extract total RNA. RNA concentrations were measured using a NanoDrop 1000 Spectrophotometer V3.2 (NanoDrop, Wilmington, DE). RNA samples were converted into first-strand cDNA with the Superscript III First-strand Synthesis System (Invitrogen, Carlsbad, CA). iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) was used to perform Real-time PCR analyses on the CFX96 real-time PCR system (Bio-Rad, Hercules, CA). The primer sequences employed are provided in Table C.1. The relative amount of the target RNA was calculated using the threshold cycle and ΔΔCt method. GAPDH was used as a reference gene to normalize expression values.

RNA isolation and whole genome microarray

RNA was extracted from cells using the RNeasy Mini RNA isolation kit (QIAGEN, Valencia, CA). RNA samples with high integrity were converted to cDNA and amplified with Applause 3'-Amp System (NuGen, San Carlos, CA). The cDNA was fragmented and Biotin labeled using the Encode Biotin Module (NuGen). Labeled cDNA was hybridized to Affymetrix HG-U133 Plus 2.0 arrays and analyzed with GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA).

Microarray data analysis

To determine differentially expressed genes in experimental miRNA and negative control treated cells, quality control was first assessed using raw CEL files as implemented in Array Analysis (Eijssen, Jaillard et al. 2013). GCRMA normalization was performed using CEL files that passed quality control. For GCRMA normalization, experimental miRNA samples (n=3 for each of miR-429 and miR-200b transfections) were independently compared to the negative control samples (n=3). Microarray Suite 5.0 (MAS5.0) from the Affymetrix Expression Console v1.1 was used to generate present/absent calls. Probe sets with less than 50% present calls across all miRNA and negative control samples and probe sets lacking a gene symbol annotation were removed. GCRMA expression signals for probe sets with the highest mean/standard deviation ratio were submitted to SAM (Significance Analysis of Microarrays) (Tusher, Tibshirani et al. 2001) and differentially expressed genes in each experimental miRNA group were determined relative to the negative control (FDR< 2%). CEL files, CHP files and GCRMA processed files are listed in the Gene Expression Omnibus under GSE56967 and GSE56969.

Cisplatin sensitivity

Quantitative assessment of cell response to cisplatin was performed as previously described (Jabbari, Reavis et al. 2014). Briefly, exponentially growing cells in 96-well plates were treated with a range of cisplatin concentrations. In order to measure the fluorescence (λex = 560 nm, λem = 590 nm), TOX-8 reagent (Sigma-Aldrich, St Louis, MO) was added to the wells after 72 hours. Outliers were removed using Grubbs' test. Growth (% of control) was determined using the ratio of the background-subtracted

fluorescence intensities of drug treated to untreated cultures was calculated in percentages. To calculate IC50 for each group, non-linear regression of log-transformed data with a normalized response-variable slope model was determined by GraphPad Prism v.6 (GraphPad Software Inc., La Jolla, CA). IC50 values are determined using at least three independent transfection experiments. In each independent experiment, triplicates of drug treated or not treated cells are used for each cisplatin concentration.

CHAPTER 5

CONCLUSIONS

MicroRNAs have recently emerged as one of the master regulators of cell function by pairing to the mRNAs of protein-coding genes to direct their repression (Bartel 2009). The mRNA target specificities of miRNAs in animals are primarily encoded within a region mapping to the molecule's 5′ end known as seed region. Most mammalian protein-coding genes are under selection to maintain pairing to the seed of one or more miRNAs (Lewis, Burge et al. , Farh, Grimson et al. 2005) illustrating the broad scope of seed-type regulation. Throughout the chapters 2 and 3, we have investigated the functional significance of sequence variation within the seed regions of miRNAs.

Results in chapter 2 are promoted by our earlier finding on the ability of miR-429 (a miR-200 family member) to induce reversal of EMT (MET) in OC mesenchymal-like cells. We systematically explored the significance of sequence variation among members of this family in OC mesenchymal-like cells. Our results indicated that even though the MET-inducing properties of miR-429 in these cells extends to other members of the miR-200 family, the underlying molecular changes are variable and attributable to sequence variation within the seed region of individual family members.

In chapter 3, in order to explore the functional significance of seed sequence variation of human miRNAs, we initially computed and compared the overlap of all conserved human miRNA predicted targets among miRNAs with identical or non-identical seed regions. The results indicated that substitution of even a single nucleotide

within the seed region dramatically changes the spectrum of miRNA targets. Next, we experimentally validated this observation by measuring the systematic effects of a set of synthetic miRNAs with non-identical seed and identical non-seed regions. The observed high functional cost of even a single nucleotide change within miRNA seed regions is suggestive of an evolutionary model whereby miRNA sequence is under stabilizing selection to maintain regulation of large suits of genes and on the other hand, lower cost associated with substitutions within the sequence of the target mRNAs allows for the evolution of their regulation by miRNAs.

Despite the importance of the seed region in miRNA targeting, recent studies indicate that pairing of seed regions with target mRNA is not always sufficient for repression(Grimson, Farh et al. 2007) and the miRNA's 3' end or the non-seed region, might also play an important role in miRNA targeting. For example, pairing to the 3′ end of the miRNA can supplement seed pairing to enhance target recognition, or it can even compensate for a single nucleotide bulge or mismatch in the seed region (Bartel 2009). Throughout the chapters 2 and 4, we provide evidence on the functional significance of sequence variation within the non-seed regions of miRNAs:

In chapter 2, consistent with the studies that have shown that OC cells induced to undergo EMT display a significantly decreased sensitivity to platinum-based drugs (Haslehurst, Koti et al. 2012, Marchini, Fruscio et al. 2013), we determined that miR-200 family induced MET in OC cells is generally correlated with a significant increase in sensitivity to cisplatin. However, significant variation was associated with sequence variation mapping to the non-seed region of individual miR-200 family members. In this case, miR-200b conferred a much higher sensitivity to cisplatin relative to miR-429 with

an identical seed region. In Chapter 4, we demonstrate that this induced increase in drug sensitivity relative to miR-429, is correlated with a significant change in global patterns of gene expression including the reduction in levels of expression of six genes previously associated with cisplatin sensitivity in a variety of cancer cells. More interestingly, both the down-regulated pattern of expression of these genes and the observed increase in cisplatin sensitivity associated with the miR-200b non-seed region were lost when a single nucleotide change was made in miR-200b's seed region. Therefore, we provide evidence that, at least in some contexts, the functional significance of the non-seed region is dependent upon the seed region with which it is paired.

The development of multicellularity is accompanied by significantly increased complexity of gene regulation. This complexity is reflected in many levels of regulation primarily at the transcription level by transcription factors (TFs) and/or at the post-transcription level by miRNAs. MiRNA mediated regulation is qualitatively different from TF mediated regulation. TFs contain discrete domains for exerting their functions, known as DNA-binding domains and protein-protein interaction domains. Because TFs have such functional units, which may individually acquire mutations and be lost or gained over time, they are modular and, thus, have opportunities to evolve in ways that minimize pleiotropy (Cheatle Jarvela and Hinman 2015). However, on the other side, miRNAs are small RNAs whose structure and function are not inherently modular. MiRNAs exert their direct regulatory function mainly through an almost perfect complementary base pairing between their seed region (in animals), or the whole miRNA sequence (in plants) and their target genes. In fact, as has been reported in this study, a single mutation in the seed region leads to a significant shift in the spectrum of miRNA

regulated genes. Therefore, any modulations in the miRNA seed are more likely to directly rewire gene expression networks and this may profoundly influence signaling pathways and serve as a base to develop malignancy. However, given that many genes in a genome are regulated by a synergistic network of miRNAs and TFs (i.e., the cascaded form, feed forward or feedback loops)(Li, Li et al. 2013), mutations in miRNA and TFs affect each other. Thus, emergence of malignancy could more be the consequence of a disrupted close synergistic regulation at transcriptional and post-transcriptional levels that is initially triggered by modulations in either TF or miRNA regulated networks.

In general, our studies support the potential therapeutic significance of groups of miRNAs and suggest that exogenous modulations in the expression of miRNAs involved in EMT may serve as the basis of important new strategies in the treatment of ovarian and other types of cancer.

APPENDIX A

SUPPLEMENTARY DATA FOR CHAPTER 3

Table A.1. Target overlap for miRNAs with identical seeds. Predicted (miRanda-mirSVR) overlap (cosine similarity) in target genes for all pairs of miRNAs with identical seed regions.

Please see table on our website:
http://www.mcdonaldlab.biology.gatech.edu/dissertations/jabbari.htm

Table A.2.Target overlap for miRNAs with non-identical seeds. Predicted (miRanda-mirSVR) overlap (cosine similarity) in target genes for all pairs of miRNAs with non-identical seed regions.

Please see table on our website:
http://www.mcdonaldlab.biology.gatech.edu/dissertations/jabbari.htm

Table A.3. Fold-change for all (5229) significantly differentially expressed genes after ectopic expression of miR-429 in HEY cells.

Please see table on our website:
http://www.mcdonaldlab.biology.gatech.edu/dissertations/jabbari.htm

Table A.4. Fold-change for all (10456) significantly differentially expressed genes after ectopic expression of M12 in HEY cells.

Please see table on our website:
http://www.mcdonaldlab.biology.gatech.edu/dissertations/jabbari.htm

Table A.5. Fold-change for all (11277) significantly differentially expressed genes after ectopic expression of M14 in HEY cells.

Please see table on our website:
http://www.mcdonaldlab.biology.gatech.edu/dissertations/jabbari.htm

Table A.6. Fold-change for all (10475) significantly differentially expressed genes after ectopic expression of M5 in HEY cells.

Please see table on our website:
http://www.mcdonaldlab.biology.gatech.edu/dissertations/jabbari.htm

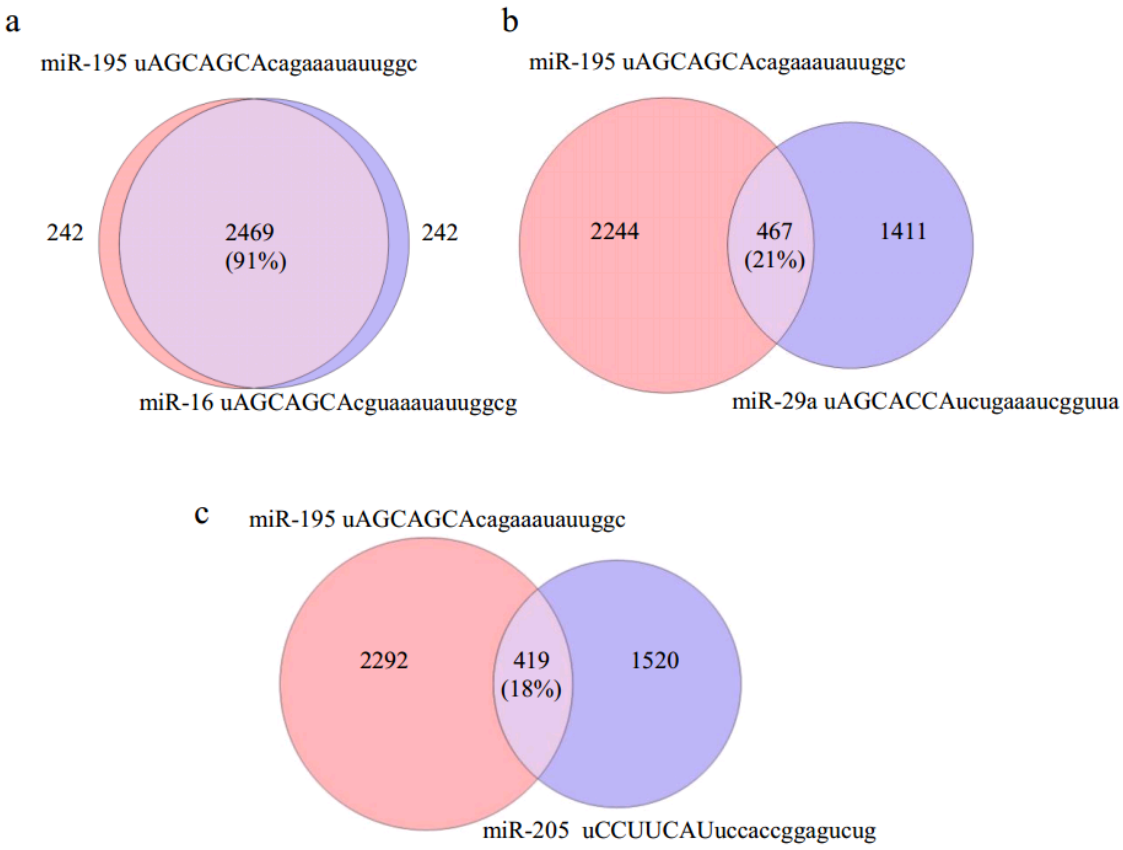


Figure A.1. One nucleotide difference or 7 nucleotide differences in the seed regions of miRNAs is predicted to be associated with equivalent levels of change in regulated target genes. (a) miRNAs with identical seeds, miR-195 and miR-16, are predicted to share 91% of their target genes; (b) miRNAs with a single nucleotide change in their seed regions, miR-195 and miR-29a, are predicted to share only 21% of their target genes; (c) miRNAs with no sequence similarity in their seed regions, miR-195 and miR-205, are predicted to share only 18% of their target genes. These results are consistent with the hypothesis that as little as a single nucleotide difference within miRNA seed regions has a major effect on miRNA regulatory control.

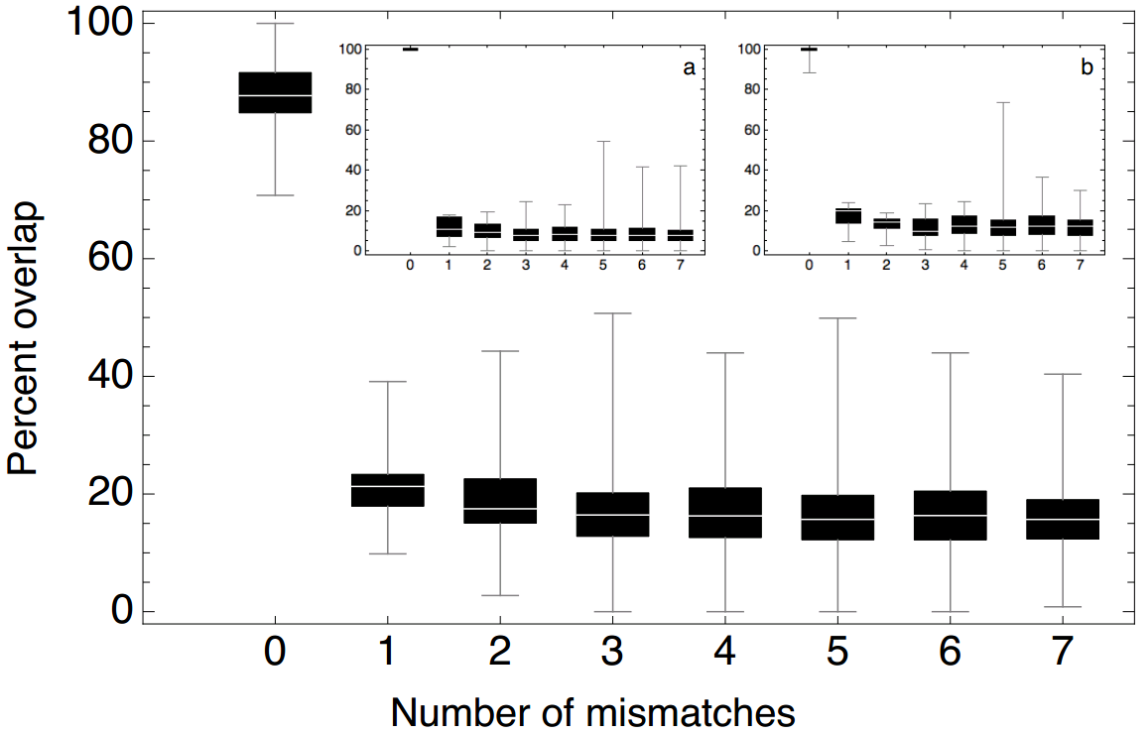


Figure A.2. Distribution of percent overlap of miRanda-mirSVR predicted targets of miRNAs having 0 through 7 seed mismatches. Insets show distribution according to two other popular prediction algorithms: a) TargetScan, and b) PicTar. The results are uniformly consistent with the prediction that even a single nucleotide mismatch within miRNA seed regions results in a large change in targeted mRNAs.

APPENDIX B

SUPPLEMENTARY DATA FOR CHAPTER 4

Table B.1. Primer sequences used for the expression measurement of the six genes

Target	Primer sequence
TYMS	Forward: 5'-CAGATTATTCAGGACAGGGAGTT-3' Reverse: 5'-CATCAGAGGAAGATCTCTTGGATT-3'
ANXA4	Forward : 5'-GGAGGTACTGTCAAAGCTGC-3' Reverse: 5'-GCCACTCAGTTCTGACTTCAG-3'
CUL4A	Forward: 5'-GGAAAGCACAGTGGTCGAA-3' Reverse: 5'-GGGACACCTGGAATTCCTTC-3'
FOXO1	Forward: 5'-GCAGATCTACGAGTGGATGGTC-3' Reverse: 5'-AAACTGTGATCCAGGGCTGTC-3'
Rac1	Forward: 5'-GCGGCACCACTGTCCCAACA-3' Reverse: 5'-AGCGCCGAGCACTCCAGGTA-3'
GSN	Forward : 5'-GGTGCAGAGACTCTTCCAGG-3' Reverse: 5'-CTGTTGGAACCACACCACTG-3'
GAPDH	Forward: 5'-TGCACCACCAACTGCTTAGC-3' Reverse: 5'-GGCATGGACTGTGGTCATGA-3'

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